Adult Muscle Progenitor Cells for Clinical Applications: Function, Safety and Interactions

---

Dissertation zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr.sc.nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
von
Meline N. L. Stölting
aus
Brasilien

Promotionskomitee
Prof. Dr. Attila Becskei, MD PhD (Vorsitz und Leitung der Promotion)
Daniel Eberli, MD PhD (Betreuung)
Prof. Tullio Sulser, MD (Betreuung)
Prof. Dr. Christoph Handschin, PhD
Dr. Stefano Ferrari, PhD

Zürich, 2013

TABLE OF CONTENTS

ZUSAMMENFASSUNG

Der Ersatz von terminal beschädigten Organen bleibt ein grosses Problem der Medizin. Der Mangel an Spenderorganen und der mit Immunsuppressions-Therapie assoziierte Komorbiditäten führen daher zur Anwendung regenerativ-medizinischer und Gewebe-züchtender Methoden im Bereich des Organersatzes. Die Verwendung von autologen Zellen und azellulärer oder synthetischer Polymere in der Rekonstruktion von Organen können helfen diese Probleme zu lösen und Patienten mit Ersatzorganen aus körpereigenen Zellen zu versorgen. Muskelstammzellen dienen die Muskelregeneration und sind deshalb eine vielversprechende Quelle für Zellmaterial. Diese Zellen beinhalten die Fähigkeit Muskelfasern zu regenerieren und wurden im Zusammenhang mit der Behandlung verschiedener Muskelkrankheiten bereits untersucht. In der Urologie eröffnen Muskelstammzellen neue Behandlungsmöglichkeiten zur Rekonstruktion von Harnblasenmuskeln, in der Behandlung sexueller Dysfunktionen und in der Behandlung von Urininkontinenzen. Urininkontinenz ist ein verbreitetes Krankheitsbild in älteren Patienten und wird gemäss der International Continence Society definiert als der unwillentliche Verlust von Urin, der zu sozialen oder hygienischen Problemen führen kann. Das Krankheitsbild betriff etwa 50% der weiblichen Population über 45 Jahren und betrifft 17% aller Männer älter als 70 Jahre. Urininkontinenz ist dennoch eine der häufigsten Komplikationen nach der Standardbehandlung für lokalisierte Prostatakarzinome und betrifft zwischen 8% und 77% der männlichen Patienten. Bevor Muskelstammzelltherapien beim Menschen angewandt werden können, müssen Funktion, Sicherheit und Interaktionen der Stammzellen nach der Implantation noch verbessert werden. Patienten, die auf gezüchtete Gewebe angewiesen sind und ihre Organe sind oft älter und haben daher ein höheres Krebsrisiko. Es war in diesem Zusammenhang bislang unbekannt, ob sich das Verhalten von Muskelstammzellen in Gegenwart von Tumoren ändert oder ob die Implantation von Muskelstammzellen in die

Nähe malignen Gewebes Tumorproliferation oder Metastasenbildung begünstigt. Ausserdem müssen Alter und Geschlecht des Donors mitberücksichtigt werden um eine dem Patienten gerechte Stammzelltherapie anbieten zu können. Ferner ist es notwendig, nach der Transplantation die weitere Zellentwicklung zu verfolgen um einen konsistenten und anhaltenden Behandlungserfolg zu erzielen. In dieser Dissertation zeigen wir, dass Muskelstammzellen generell sicher sind bei der Muskelregeneration bei Patienten die zuvor an Karzinomen litten. Muskelstammzellen verhindern ein erneutes Auftreten von Karzinomen indem sie parakrines TNFα sekretieren und das Tumorwachstum inhibieren. Solche Muskelstammzellen können von Patienten beiderlei Geschlechts und aller Altersgruppen isoliert werden und tragen bei, neues Muskelgewebe mit sich verbessernder Funktion zu bilden. Das hohe Wachstumspotential von Muskelstammzellen und ihre Funktionalität erlauben die Transplantation hinreichend vieler Zellen schon 3 Wochen nach der Muskelbiopsie. Zusätzlich kann die Zellintegration und Muskelregeneration nach der Muskelstammzellimplantation durch magnetische Stimulation angeregt werden, die das Einwachsen von Nerven und die Ausbildung Neuromuskulärer Junctions begünstigt. Diese nicht-invasive und durch die FDA anerkannte Behandlungsmethode kann also benutzt werden um die Bildung von Muskelfasern und die Gewebeintegration zu verbessern.

SUMMARY

The replacement of terminally damaged organs remains a major problem in healthcare. The shortage of available donor organs and the high morbidity of immunosuppressive therapy lead to the application of regenerative medicine and tissue engineering to the field of organ replacement. The use of autologous cells and acellular or synthetic polymers for organ reconstruction has the potential to overcome these shortcomings and provide replacement organs made from patients own cells. Muscle Precursor Cells (MPCs), for muscle regeneration, are envisioned as promising cell sources with the capability to regenerate muscle fibers, and therefore investigated for the treatment of several muscular diseases. In Urology, it opens novel treatment possibilities including reconstruction of bladder muscles, management of sexual dysfunction and treatment of Urinary Incontinence. Urinary Incontinence is a common condition in the elderly defined by the International Continence Society (ICS) as an involuntary loss of urine leading to social or hygienic problems[1]. It affects around half of the female population[2] over 45 years and 17% of men after 70 years[3]. In men, urinary incontinence is additionally one of the most frequent complications of the standard therapy to localized prostate carcinoma, with incidence ranging between 8 to 77%[4]. MPCs safety and interactions need to be investigated and its function after implant needs to be improved previous to human application. Patients in need of engineered tissues and organs are older and therefore exposed or at risk of cancers. However, it was hitherto unknown whether the behavior of MPC changes on the presence of tumor or if implanting MPC in the proximity of malignant tissues may induce tumor proliferation and metastasis. Likewise, age and gender of donor and recipient need to be taken into consideration to develop a cell therapy that reaches the specific patient needs. Moreover, a cell follow-up after transplantation is necessary to ensure a consistent and long-lasting therapeutic benefit. In this thesis when report that MPCs provide an overall safe muscle regeneration even for patients with previous cancer. They prevent cancer recrudescence by

secreting paracrine TNFα and inhibiting tumor growth. Also, these cells can be isolated from patients of all ages and both sex, forming new muscle tissue with time progressing function. The growth potential of MPCs and function output after transplantation permits autologous transplantation of sufficient cell numbers 3 weeks after muscle biopsy. Additionally, magnetic stimulation supports cell integration and muscle regeneration after MPC implantation, promotes nerve ingrowth and the development of organized neuromuscular junctions. This non-invasive FDA approved treatment modality can be used to further improve muscle fiber formation and tissue integration.

INTRODUCTION

CELL THERAPY FOR MUSCLE REGENERATION*

Comprising nearly 50% of the human body[5] skeletal muscles compose the machinery that sets the body in movement. When well-trained they have the capability to protect joints and bones from daily waste and trauma[6]. They hold an intrinsic protective mechanism against cancer formation and metastasis settling[7] and are at the same time the main energy reservoir of the body storing more than 80% of our reserve glycogen[8]. Above and beyond that skeletal muscle upholds powerful stem cells that enable skeletal muscle to display an astonishing regenerative capacity[9]. Due to these resident muscle progenitor cells (MPCs), after one week of severe trauma new myotubes are already being formed, and within 28 days muscle regeneration after trauma is almost complete[10]. This mosaic of intrinsic features makes of skeletal muscle a very interesting site of study.

SCs are the secret of Skeletal muscle regeneration

The secret of skeletal muscle staggering regenerative capacity is found in the specific components of its cell niche. The muscle niche is composed of long and slender cells that form muscle fibers grouped in bundles (Figure 1). Adjacent to these myofibers, a heterogeneous pool of subsarcolemmal progenitor and stem cells respectively committed to myogenic differentiation or to self-renewal, known as muscle satellite cells (SC), guaranty a fast and efficient regenerative process after trauma [11]. These cells activated by injury[12] work hierarchically to maintain the *in situ* pool of cells (Figure 1) and to reconstruct the damaged tissue in less than one month by differentiating into new myotubes.

*Partially published as a book chapter in ***Regenerative Medicine and Tissue Engineering***.

Injury and Inflammation – the role of inflammation

After trauma an inflammatory infiltrate takes place and four different types of cells namely, neutrophils, macrophages, satellite cells and myoblasts work chronologically together cleaning up damaged fibers and reconstructing new functional myotubes. Neutrophils are the first cells to arrive the injury site followed by macrophages after 3 hours of damage[10]. Through the combined action of free radicals, growth factor and chemotactic factors these inflammatory cells contribute both to injury and repair[13]. Without the neutrophils- related oxidative and proteolytic modifications of damaged tissue, phagocytosis of debris is not possible[14]. Along with it, the macrophages are the major housecleaners that remove remaining debris of fiber and picnotic cells. Furthermore, macrofages produce proteases to lyse the sarcolemmal membrane, what allows activation and proliferation of SC[15]. Dismantling the extracellular matrix is key to SC activation, and the up-regulation of metalloproteinases is required to muscle regeneration[16]. Macrophages infiltrate is also important to satellite cell activation and proliferation by activating NF-$_{\kappa}$B via TWEAK ligand. [17].

Quiescent SCs are still found between the basal membrane and sarcolemma until the third day after injury. Subsequent, they are slowly replaced by cells with large nuclei, nucleoli, and cytoplasmatic processes filled with ribonucleoprotein granules. These myoblasts display an initial exponential growth phase but from the seven day onward they start to form myotubes with centrally placed nuclei and peripheral myofibrils. On the periphery of these newly formed myotubes, already on the 8th day after trauma, a new population of subsarcolemmal quiescent cells replenish the SC pool[10]. Finally, myofibers nuclei do not display mitotic figures throughout the regeneration process, demonstrating that the damaged fiber cannot heal itself without the activation of satellite cells.

The role of the muscle niche on muscle regeneration

Components of the muscle niche are also important to skeletal muscle regeneration and satellite cell activation. The basal lamina is the common anatomic site of satellite cells and also contributes to cell fate. The basal lamina is rich in α7β1 integrin which act directly in the anchorage, adhesion and quiescence of satellite cells[18]. These integrin functions also comprise the migration and proliferation of developing myoblasts[19], the formation and integrity of neuromuscular junctions[20], as well as the binding of muscle fibers. Another integrin, VLA-4, is expressed as myotubes form and influence the alignment and fusion of myoblasts[21]. Finally, the calcium-dependent cell adhesion protein M-cadherin is a morphoregulatory molecule facilitating myoblasts fusion and cell adhesion to its adjacent myofiber[22, 23].

The surrounding acellular matrix (ACM) contains a list of components that can influence the behavior and regulate the growth of muscle progenitor cells. The ACM is a source of hepatocyte[24] and fibroblast[25] growth factors, which act respectively on the activation of satellite cells, proliferation and inhibition of differentiation. Endothelial growth factor is also produced by the ACM which promotes satellite cell activation and survival after injury[26]. Finally, the aged ACM is capable of impairing the regenerative potential of satellite cells and inducing fibrosis by activating the canonical Wnt signaling pathway[27].

Fibroblasts are the main source of collagen into the muscular interstitial space[28]. They continuously promote the formation of the basal lamina during myogenesis[29] and after muscle injury proliferate hand in hand with $Pax7^+$ satellite cells orchestrating the fine balance between muscle reconstruction and fibrosis formation[30]. These $TCF4^+$ fibroblasts prevent premature activation and differentiation of $Pax7^+$ progenitor cells, what avoids the depletion of the pool of satellite cells and myoblasts. Accordingly, satellite cells are sufficient to regulate the ingrowth of fibroblasts and fibrosis formation[30]. They are also involved in myosin switch from fetal to adult muscle, specially promoting Myosin Heavy Chain type 1 expression

(slow twitch) in several limb muscles in the fetal mouse and in the soleus in the adult muscle[31].

Circulating and locally produced soluble factors participate on the signaling pathway that regulates satellite cell activity. During exercise and stretching muscle fibers liberate HGF through nitric oxide stimulation and induce activation of satellite cells[32]. HGF can also activate satellite cells by activating the sphingolipid signaling cascade upon disruption of the laminin-integrin adhesion on the event of trauma[33]. Furthermore, the insulin-like growth factor 1 (IGF-1), a potent mitogen produced locally during muscle hypertrophy and injury, can also induce activation, proliferation and differentiation of satellite cells[12, 34]. Contrasting, the growth differentiation factor member of the TGF-beta protein family myostatin secreted by adult skeletal muscle is capable of inhibiting activation and self-renewal of quiescent cells [35]. Finally, a hormone produced by the thyroid gland and responsible for inducing hypercalcemia named Calcitonin [36], has been associated to delay on satellite cell activation [37]. Altogether these components and products of the muscle niche are key regulators of all the process of development and regeneration of skeletal muscle.

Satellite cells are also required for exercise related muscle turn-over

Exercise is capable of activating muscle gene transcription within seconds and these molecular responses can last hours even after exercise cessation[38]. During endurance exercise, muscle consume large amounts of oxygen to generate energy through carbohydrates and posteriorly fat breaking down[39]. Muscle fibers are not in a smooth continuous muscle contraction during exercise, but rather act as a series of small groups of fibers contracting at the same moment[40]. This occurs due to stimulation of neuromuscular junction by terminal branches of axons whose cell body is in the anterior horn of the spinal cord. Altogether, this nerve and muscle components comprise the motor unit[41] and conduce impulses that enable sharp muscle contraction within milliseconds[42]. A signaling pathway is then activated by

rapamycin kinase (mTOR) leading to hypertrophic changes on muscle mass[43]. The opposing effect is found during starvation when the AMP-activated protein kinase (AMPK) is switched on to up-regulate energy-conserving processes and ultimately induce muscle atrophy[43]. However, exercise is sufficient to increase the pool of stem cells reversing the effects of atrophy after prolonged limb immobilization[44].

In the onset of trauma or during exercise nitric oxide is liberated and modulated the activation of satellite cells[45, 46]. Myonuclear accretion, the increase of myonuclei inside of myofibers caused by satellite cells proliferation and fusion, is detected after muscle functional overload and exercise[47]. One acute bout only of resistance exercise is already enough to cause satellite cell activation[48]. Another evidence of this cell addition during exercise is the detected decrease of telomeres length in marathon runners, which correlates to their running hours[49]. It is described that endurance exercise stimulates the production of free radicals like nitric oxide [50], which has been described to induce the activation of satellite cells increasing muscle turn-over[32]. In the other hand, during muscle atrophy process caused by limb immobilization a apoptotic decrease on myonuclei occurs[51] associated with drop on satellite cells mitotic activity[52]. These findings suggest the involvement of satellite cells in the regulation of muscle mass during exercise.

Markers for satellite cells

A transcriptional network controls progression of both embryonic and adult muscle stem cell[53]. Muscle embryonic progenitor cells can be identified (Figure 2) by the co-expression of the paired-domain transcription factors Pax3 and Pax7 and are maintained as a self-renewing proliferative population[54]. During embryogenesis Pax3 is required to maintain muscle progenitor cells in the somite and further induce cell migration to the required site of skeletal myogenesis[55]. Indeed the normal expression of Pax3 seems to be decisive to the development of normal muscle, and its mutation promotes malignant growth and induces tumorigenesis in

alveolar rhabdomyosarcoma tumor cells[56]. However, its down-regulation is necessary to final cell commitment to myogenesis and leads to rapid and robust entry into the myogenic differentiation program[55]. The expression of the transcription factor Pax7 is detectable from the embryonic muscle progenitor until the quiescent and activated satellite cells (Figure2). Its induction in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs [57].

Specific molecular markers have been demonstrated to distinguish between activated and quiescent SC. Quiescent satellite cells express the transcription factor Pax7 and when activated, coexpress Pax7 with MyoD [58]. This dual expression is followed by a proliferate phase, down-regulation of Pax7 and terminal differentiation. If Pax3 and Pax7 down-regulation do not occur into the injury site and *in vitro* an evident side effect will be the blockage of differentiation[59, 60]. In these context microRNAs (miRNAs) play a regulatory role conferring robustness to developmental timing by posttranscriptional repression of genetic programs of progenitor and satellite cells[61]. They allow rapid gene program transitions from proliferation to differentiation, blocking PAX3[62] and Pax7[63] activity on progenitor and satellite cells.

This interplay during development is required to ignite the satellite cells commitment to myogenic program, activate the myogenic regulatory factors Myf-5 and MyoD and promote terminal muscle differentiation[61]. In fact, changes in miRNAs levels are accompanied by endogenous alterations, leading to a MyoD induced repression of Pax3 expression[64] and regulation of cell cycle components[65], which are decisive to subsequent myoblast cell cycle progression or exit into differentiation. Through the action of the myogenic regulatory factors (MRFs), Myf5 and MyoD the muscle progenitor cells ($Pax3^+$) and quiescent satellite cells ($Pax3^+/Pax7^+$) become muscle lineage committed and activated myoblasts[66]. They express *Myf5* and *Mrf4* and rapidly give rise to $Desmin^+$ cells, which differentiation is regulated by

myogenin, MyoD and MRF4[67]. Completing these regulatory features, MyoD is also a main player in the intricate epigenetic cascade that controls skeletal myogenesis[68].

Cell delivery – Cell Carrier

The ability to regenerate muscle tissue from patients own cells would have profound impact on many human diseases. Cell therapy is within reach as a novel treatment option for incontinence, reflux, vocal cord dysfunction and other muscle-related pathologies. However, the carrier used for cell delivery and the techniques used to non-invasively define the needing site for cell delivery are still being optimized. It has been demonstrated for more than a decade that cells injected in a saline solution carrier are able to ectopically form contractile muscle [69]. However, further studies have reported very poor survival rates (5-20%) associated with myogenic cell implantation without embedding into protein based carriers that support cell settling into their new niche [70, 71].

Species-specific cues play an important role on cell affinity to carriers. A previous study demonstrated advantage of using collagen rather than matrigel coated dishes on boosting cell growth and differentiation potential [72]. In contrast, another study with porcine satellite cells demonstrated cell preference to matrigel coated dishes and growth decrease on collagen layers[73]. Moreover, three-dimensional(3D) matrigel coated PLGA scaffolds (poly lactic-co-glycolic acid) were capable of improving cell survival when compared to direct cell injection[74]. However, the same study failed to demonstrate a comparative improvement of matrigel coated PLGA with other cell carriers. Furthermore, matrigel has not presented advantage *in vivo a*s a carrier for myogenic cells when compared to hyaluronic acid-photoinitiator (HA-PI) complex. It rather downgraded the quality of muscle structure and decreased the total number of new myofibers after cell injection [75].

Collagen is a main component of the natural extracellular matrix of skeletal muscle, it is therefore expected that satellite cells would have their functionality up-scaled in a collagen

rich environment[76]. Combined with electrical stimulation it induces 3 dimensional expansion of muscle precursor cells *in vitro* and in syngeneic recipient muscle [77]. Cell cycle analyses of implanted engrafts into a 3D collagen sponge highlighted the increment of cell fractions in proliferating phases, with 80% of cell survival[78]. Furthermore, the use of parallel aligned collagen nanofiber yielded good proliferation and enabled the generation of aligned cell layers[79]. Finally, grafts of myoblasts seeded into three-dimensional collagen scaffold and implanted into induced defect sites in mice demonstrated improvement in muscle healing, innervation and vascularization[80]. Altogether these recent studies confirm that collagen is a very promising matrix for satellite cells ingrowth and an ideal carrier for the transplantation of myogenic cells.

Cell Delivery – Imaging Techniques for guided cell implantation in vivo

The success of cell transplantation into a specific site *in vivo* is directly dependent of 3 key points: cell source, cell carrier and injection technique. The two first were previously discussed on this chapter. We dedicate this section to the discussion of the injection techniques that were so far used to inject myogenic cells into a specific injury site. The application of myogenic cells was already used for the treatment of male and female individual with urinary incontinence, the involuntary loss of urine that represents a hygienic and social problem[1]. Transurethral ultrasound guided injections of autologous cells isolated from limb skeletal muscle biopsies were so far the method of choice [81, 82]. This method is also standard for the injection of bulking agent like collagen in the clinic practice[83]. Finally, ultrasound guidance was also used to monitor percutaneous trans-coronary-venous transplantation of autologous myoblasts in infarcted myocardium[84, 85].

Recently magnetic resonance imaging (MRI) raises attention as a useful tool to guidance during injection of drugs and potentially of cells[86]. Pulsed focused ultrasound is a new ultrasound technique that associated with magnetic resonance guidance was recently

suggested as a new imaging modality that may be utilized to target cellular therapies by increasing homing to areas of pathology[87]. It was also being demonstrated to increase drug uptake into a specific target in the prostate[88] and brain[89]. This same technique has been demonstrated to facilitate the delivery of neural stem cells into a specific site in the brain[90]. Overall, the most successful delivery of myogenic cells have been done either operatively in 3D scaffolds or into collagen carrier that facilitates cell settling into the new cell niche. For clinical application imaging techniques need to be used as transplant guidance and will vary according to the recipient site. To do so ultrasonography is still the most adaptable and widely used imaging technique. However, new approaches combining MRI and ultrasonographic pulses are very promising methods that need to be further studied and adapted to cell injection in different anatomic sites.

Overcoming pitfalls on stem cell therapy – Safety, the role of cell donors and long term cell survival and function after transplantation

A decline of approximate 30% in muscle strength and 40% in muscle area occurs between the second and seventh decades of life[91]. Also the total number of MPCs and their proliferation potential in culture gradually decrease in an age-dependent manner [92] due to apoptosis [93]. Additionally cell fate is tightly defined by the interactions with the microenvironment and the host age is of key importance, as the stem cell regenerative capacity reduces in aged niches [94]. We have studied the influence of age and gender on the growth and function of MPCs. We report that although human MPCs can be successfully isolated and grown from patients of all ages and genders (figure 3), both elderly and male donors provide unstable and slower growing cells *in vitro* with decreased contractile output *in vivo*[95]. Female and young cells were capable of preserving their stem-likeness for longer time in culture and after transplantation differentiated into organized and stronger muscle fibers.

While elderly patients are the main patient group that would benefit from MPCs therapy, they are also at higher risk of cancer. In fact, the need of a urinary sphincter reconstruction in man would mainly be present after a prostatectomy, which is the operative extraction of a cancerous prostate. This leads to the need of an investigation of the safety of MPCs in the proximity of cancer. In this thesis we report that muscle cell therapy is safe and has the potential to reduce tumor recrudescence [7]. Those cells were capable to grow and differentiate in the proximity of cancer without altering their muscle phenotype. At the same time, MPCs secreted paracrine TNFα, which induced cancer cell cycle arrest and apoptosis *in vitro* and *in vivo*.

Knowing that stem cell therapy is safe leads to the question of the long term survival and function of newly formed tissue. To provide a long-lasting muscle function not only the muscle component, provided by the MPCs injection, is necessary but also a functional innervation of the engineered tissue. Although innervation of the newly implanted tissue is also essential to engineer a functional muscle tissue there is few approaches that could effectively promote nerve ingrowth after transplantation. Some studies described a spontaneous nerve ingrowth from the neighbor tissues into the newly transplanted sites [96, 97], but noninvasive methods to induce nerve ingrowth after newly formed muscle engrafts are still to be investigated. We report that magnetic stimulation supports regeneration of injured muscle with activating resident stem cells or supporting integration of newly implanted myoblasts. Exposition of injured limb and co-cultures of muscle cells and neurons to magnetic fields was sufficient to trigger synapses, induce acetylcholine receptors clustering and cause typical muscular metabolic adaptations verified during endurance exercise. Notwithstanding, magnetic stimulation mimicked the effects of exercise inducing PGC1α up-regulation, induces myogenic cells differentiation and increases nerve fibers and acetylcholine receptor clustering after cell transplantation. New efforts in establishing functional innervation, proper vascular network and the development of a high endurance resistance

muscle are going to be decisive the three main pillars supporting future translational studies and bringing myogenic cell transplantation from bench to bedside.

Future approaches - reactivating muscle metabolism, and tissue vascularization and innervation

In the context of muscle reconstruction, gene therapy is not aimed at rectifying a genetic mutation, but at boosting the myogenic potential and ultimately the muscle functionality of the injected autologous muscle cells. Two key factors have been demonstrated to improve the quality of satellite cells for transplantation: a better vascularization[98] and endurance exercise[99]. We have previously described that an angiogenic modification of muscle precursors can overcame some of the limitations of old muscle stem cells[98]. For future application expanding the knowledge produced on this study and therapeutically combining it with the intrinsic adaptation effects of endurance exercise would be of major interest. On this context, studies using muscle-specific PGC-1α transgenic animals demonstrated that ectopic expression of PGC-1α in muscle seems sufficient to evoke a trained phenotype avoiding muscle atrophy [100]. Upon activation, PGC-1α in turn controls many, if not all of the adaptations of skeletal muscle to endurance exercise [101]. Hereafter, PGC-1α muscle-specific transgenic animals exhibit high endurance, oxidative muscle fibers, an increase in mitochondrial biogenesis and oxidative metabolism, augmented muscle capillarization and a remodeling of the neuromuscular junction [102, 103].

REFERENCES

1. Abrams, P. *et al.* The standardisation of terminology of lower urinary tract function: Report from the standardisation sub-committee of the International Continence Society. *Neurourology and Urodynamics* **21**, 167-178 (2002).
2. Swanson, J., Kaczorowski, J., Skelly, J. & Finkelstein, M. Urinary incontinence: common problem among women over 45. *Can Fam Physician* **51**, 84-85 (2005).
3. Molander, U., Sundh, V. & Steen, B. Urinary incontinence and related symptoms in older men and women studied longitudinally between 70 and 97 years of age. A population study. *Archives of Gerontology and Geriatrics* **35**, 237-244 (2002).
4. Klingler, H.C.M., Michael Incontinence after radical prostatectomy: surgical treatment options. *Current Opinion in Urology* **16**, 60-64 (2006).
5. Surov, A. *et al.* Skeletal muscle metastases: primary tumours, prevalence, and radiological features. *European Radiology* **20**, 649-658 (2010).
6. Rose, S.J. & Rothstein, J.M. Muscle Mutability. *Physical Therapy* **62**, 1773-1787 (1982).
7. Stölting, M.N.L. *et al.* Muscle Precursor Cells inhibit tumor growth upon secretion of TNF alpha. *Journal of Urology* **in print** (2012).
8. Jensen, J.r., Rustad, P.I., Kolnes, A.J. & Lai, Y.-C. The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers in Physiology* **2** (2011).
9. Relaix, F. & Zammit, P.S. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139**, 2845-2856 (2012).
10. Church, J.C.T., Noronha, R.F.X. & Allbrook, D.B. Satellite cells and skeletal muscle regeneration. *British Journal of Surgery* **53**, 638-642 (1966).
11. Kuang, S., Gillespie, M.A. & Rudnicki, M.A. Niche Regulation of Muscle Satellite Cell Self-Renewal and Differentiation. *Cell stem cell* **2**, 22-31 (2008).
12. Hill, M., Wernig, A. & Goldspink, G. Muscle satellite (stem) cell activation during local tissue injury and repair. *Journal of Anatomy* **203**, 89-99 (2003).
13. Tidball, J.G. Inflammatory processes in muscle injury and repair. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **288**, R345-R353 (2005).
14. Teixeira, C.F.P. *et al.* Neutrophils do not contribute to local tissue damage, but play a key role in skeletal muscle regeneration, in mice injected with Bothrops asper snake venom. *Muscle & Nerve* **28**, 449-459 (2003).
15. Tidball, J.G. & Wehling-Henricks, M. Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *The Journal of Physiology* **578**, 327-336 (2007).
16. Pallafacchina, G. *et al.* An adult tissue-specific stem cell in its niche: A gene profiling analysis of in vivo quiescent and activated muscle satellite cells. *Stem Cell Research* **4**, 77-91 (2010).
17. Girgenrath, M. *et al.* TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. *EMBO J* **25**, 5826-5839 (2006).
18. Burkin, D.J. & Kaufman, S.J. The a7ß1 integrin in muscle development and disease. *Cell and Tissue Research* **296**, 183-190 (1999).
19. Liu, J., Burkin, D.J. & Kaufman, S.J. Increasing α7β1-integrin promotes muscle cell proliferation, adhesion, and resistance to apoptosis without changing gene expression. *American Journal of Physiology - Cell Physiology* **294**, C627-C640 (2008).
20. Nishimune, H. *et al.* Laminins promote postsynaptic maturation by an autocrine mechanism at the neuromuscular junction. *The Journal of Cell Biology* **182**, 1201-1215 (2008).

21. Rosen, G.D. *et al.* Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. *Cell* **69**, 1107-1119 (1992).
22. Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A. & Wernig, A. Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Developmental Dynamics* **199**, 326-337 (1994).
23. Dottermusch-Heidel, C., Groth, V., Beck, L. & Önel, S.-F. The Arf-GEF Schizo/Loner regulates N-cadherin to induce fusion competence of Drosophila myoblasts. *Developmental Biology* **368**, 18-27 (2012).
24. Tatsumi, R., Anderson, J.E., Nevoret, C.J., Halevy, O. & Allen, R.E. HGF/SF Is Present in Normal Adult Skeletal Muscle and Is Capable of Activating Satellite Cells. *Developmental Biology* **194**, 114-128 (1998).
25. DiMario, J., Buffinger, N., Yamada, S. & Strohman, R.C. Fibroblast Growth Factor in the Extracellular Matrix of Dystrophic (mdx) Mouse Muscle. *Science* **244**, 688-690 (1989).
26. Golding, J.P., Calderbank, E., Partridge, T.A. & Beauchamp, J.R. Skeletal muscle stem cells express anti-apoptotic ErbB receptors during activation from quiescence. *Experimental Cell Research* **313**, 341-356 (2007).
27. Brack, A.S. *et al.* Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate and Increases Fibrosis. *Science* **317**, 807-810 (2007).
28. Zou, Y., Zhang, R., Sabatelli, P., Chu, M. & Bönnemann, C. Muscle Interstitial Fibroblasts Are the Main Source of Collagen VI Synthesis in Skeletal Muscle: Implications for Congenital Muscular Dystrophy Types Ullrich and Bethlem. *J Neuropathol Exp Neurol* (2008).
29. Sanderson, R.D., Fitch, J.M., Linsenmayer, T.R. & Mayne, R. Fibroblasts promote the formation of a continuous basal lamina during myogenesis in vitro. *The Journal of Cell Biology* **102**, 740-747 (1986).
30. Murphy, M.M., Lawson, J.A., Mathew, S.J., Hutcheson, D.A. & Kardon, G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* **138**, 3625-3637 (2011).
31. Mathew, S.J. *et al.* Connective tissue fibroblasts and Tcf4 regulate myogenesis. *Development* **138**, 371-384 (2011).
32. Tatsumi, R. *et al.* Satellite cell activation in stretched skeletal muscle and the role of nitric oxide and hepatocyte growth factor. *American Journal of Physiology - Cell Physiology* **290**, C1487-C1494 (2006).
33. Macaluso, F. & Myburgh, K. Current evidence that exercise can increase the number of adult stem cells. *Journal of Muscle Research and Cell Motility* **33**, 187-198 (2012).
34. Kumar, A., Yamauchi, J., Girgenrath, T. & Girgenrath, M. Muscle-specific expression of insulin-like growth factor 1 improves outcome in Lama2Dy-w mice, a model for congenital muscular dystrophy type 1A. *Human Molecular Genetics* **20**, 2333-2343 (2011).
35. Joulia-Ekaza, D. & Cabello, G. The myostatin gene: physiology and pharmacological relevance. *Current Opinion in Pharmacology* **7**, 310-315 (2007).
36. Becker, K.L., Nylén, E.S., White, J.C., Müller, B. & Snider, R.H. Procalcitonin and the Calcitonin Gene Family of Peptides in Inflammation, Infection, and Sepsis: A Journey from Calcitonin Back to Its Precursors. *Journal of Clinical Endocrinology & Metabolism* **89**, 1512-1525 (2004).
37. Fukada, S.-i. *et al.* Molecular Signature of Quiescent Satellite Cells in Adult Skeletal Muscle. *Stem Cells* **25**, 2448-2459 (2007).
38. Neufer, P.D., Ordway, G.A. & Williams, R.S. Transient regulation of c-fos, αB-crystallin, and hsp70 in muscle during recovery from contractile activity. *American Journal of Physiology - Cell Physiology* **274**, C341-C346 (1998).

39. Shefer, G. & Benayahu, D. The effect of exercise on IGF-I on muscle fibers and satellite cells. *Front Biosci* **E4**, 203-239 (2012).
40. Harber, M. & Trappe, S. Single muscle fiber contractile properties of young competitive distance runners. *Journal of Applied Physiology* **105**, 629-636 (2008).
41. Bogdanis, G.C. Effects of physical activity and inactivity on muscle fatigue. *Frontiers in Physiology* **3** (2012).
42. Nakayama, T. & Hori, T. Conduction Velocity of the Tonic Motor Fibers in Man. *The Tohoku Journal of Experimental Medicine* **93**, 391-394 (1967).
43. Mounier, R. *et al.* Antagonistic control of muscle cell size by AMPK and mTORC1. *Cell Cycle* **10**, 2640-2646 (2011).
44. Shefer, G., Carmeli, E., Rauner, G., Yablonka-Reuveni, Z. & Benayahu, D. Exercise running and tetracycline as means to enhance skeletal muscle stem cell performance after external fixation. *Journal of Cellular Physiology* **215**, 265-275 (2008).
45. Soltow, Q. *et al.* Nitric oxide regulates stretch-induced proliferation in C2C12 myoblasts. *Journal of Muscle Research and Cell Motility* **31**, 215-225 (2010).
46. Leiter, J.R.S. & Anderson, J.E. Satellite cells are increasingly refractory to activation by nitric oxide and stretch in aged mouse-muscle cultures. *The International Journal of Biochemistry & Cell Biology* **42**, 132-136 (2010).
47. Pallafacchina, G., Blaauw, B. & Schiaffino, S. Role of satellite cells in muscle growth and maintenance of muscle mass. *Nutrition, Metabolism and Cardiovascular Diseases*.
48. Walker, D.K. *et al.* PAX7+ satellite cells in young and older adults following resistance exercise. *Muscle & Nerve* **46**, 51-59 (2012).
49. Rae, D. *et al.* Skeletal muscle telomere length in healthy, experienced, endurance runners. *European Journal of Applied Physiology* **109**, 323-330 (2010).
50. Sachdev, S. & Davies, K.J.A. Production, detection, and adaptive responses to free radicals in exercise. *Free Radical Biology and Medicine* **44**, 215-223 (2008).
51. Hikida, R.S. *et al.* Myonuclear loss in atrophied soleus muscle fibers. *The Anatomical Record* **247**, 350-354 (1997).
52. Schultz, E., Darr, K.C. & Macius, A. Acute effects of hindlimb unweighting on satellite cells of growing skeletal muscle. *Journal of Applied Physiology* **76**, 266-270 (1994).
53. Parker, M.H., Seale, P. & Rudnicki, M.A. Looking back to the embryo: defining transcriptional networks in adult myogenesis. *Nat Rev Genet* **4**, 497-507 (2003).
54. Amthor, H., Christ, B. & Patel, K. A molecular mechanism enabling continuous embryonic muscle growth - a balance between proliferation and differentiation. *Development* **126**, 1041-1053 (1999).
55. Crist, C.G. *et al.* Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. *Proceedings of the National Academy of Sciences* **106**, 13383-13387 (2009).
56. Keller, C. & Capecchi, M.R. New Genetic Tactics to Model Alveolar Rhabdomyosarcoma in the Mouse. *Cancer Research* **65**, 7530-7532 (2005).
57. Seale, P. *et al.* Pax7 Is Required for the Specification of Myogenic Satellite Cells. *Cell* **102**, 777-786 (2000).
58. Zammit, P.S. *et al.* Pax7 and myogenic progression in skeletal muscle satellite cells. *Journal of Cell Science* **119**, 1824-1832 (2006).
59. Montarras, D. *et al.* Direct Isolation of Satellite Cells for Skeletal Muscle Regeneration. *Science* **309**, 2064-2067 (2005).
60. Olguin, H.C. & Olwin, B.B. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Developmental Biology* **275**, 375-388 (2004).

61. Koutsoulidou, A., Mastroyiannopoulos, N., Furling, D., Uney, J. & Phylactou, L. Expression of miR-1, miR-133a, miR-133b and miR-206 increases during development of human skeletal muscle. *BMC Dev Biol* **11**, 1-9 (2011).
62. Goljanek-Whysall, K. *et al.* MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis. *Proceedings of the National Academy of Sciences* **108**, 11936-11941 (2011).
63. Chen, J.-F. *et al.* microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *The Journal of Cell Biology* **190**, 867-879 (2010).
64. Hirai, H. *et al.* MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. *The Journal of Cell Biology* **191**, 347-365 (2010).
65. Kitzmann, M. & Fernandez, A. Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. *Cellular and Molecular Life Sciences (CMLS)* **58**, 571-579 (2001).
66. Tajbakhsh, S. & Buckingham, M. The birth of muscle progenitor cells in the mouse: Spatiotemporal considerations. *Current Topics in Developmental Biology, Vol 48* **48**, 225-268 (2000).
67. Valdez, M.R., Richardson, J.A., Klein, W.H. & Olson, E.N. Failure of Myf5 to Support Myogenic Differentiation without Myogenin, MyoD, and MRF4. *Developmental Biology* **219**, 287-298 (2000).
68. Saccone, V. & Lorenzo Puri, P. Epigenetic regulation of skeletal myogenesis. *Organogenesis* **6**, 48-53 (2010).
69. Irintchev, A., Rosenblatt, J.D., Cullen, M.J., Zweyer, M. & Wernig, A. Ectopic skeletal muscles derived from myoblasts implanted under the skin. *Journal of Cell Science* **111**, 3287-3297 (1998).
70. Qu, Z. *et al.* Development of Approaches to Improve Cell Survival in Myoblast Transfer Therapy. *The Journal of Cell Biology* **142**, 1257-1267 (1998).
71. Holzer, N. *et al.* Autologous transplantation of porcine myogenic precursor cells in skeletal muscle. *Neuromuscular Disorders* **15**, 237-244 (2005).
72. Eberli, D., Soker, S., Atala, A. & Yoo, J.J. Optimization of human skeletal muscle precursor cell culture and myofiber formation in vitro. *Methods* **47**, 98-103 (2009).
73. Grefte, S., Vullinghs, S., Kuijpers-Jagtman, A.M., Torensma, R. & Hoff, J.W.V.d. Matrigel, but not collagen I, maintains the differentiation capacity of muscle derived cells in vitro. *Biomedical Materials* **7**, 055004 (2012).
74. Boldrin, L. *et al.* Satellite Cells Delivered by Micro-Patterned Scaffolds: A New Strategy for Cell Transplantation in Muscle Diseases. *Tissue Engineering* **13**, 253-262 (2007).
75. Rossi, C.A. *et al.* In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. *The FASEB Journal* **25**, 2296-2304 (2011).
76. Sharples, A.p. *et al.* Modelling in-vivo skeletal muscle ageing in-vitro using three dimensional bioengineered constructs. *Aging Cell*, no-no (2012).
77. Serena, E. *et al.* Electrophysiologic stimulation improves myogenic potential of muscle precursor cells grown in a 3D collagen scaffold. *Neurological Research* **30**, 207-214 (2008).
78. Flaibani, M., Luni, C., Sbalchiero, E. & Elvassore, N. Flow cytometric cell cycle analysis of muscle precursor cells cultured within 3D scaffolds in a perfusion bioreactor. *Biotechnology Progress* **25**, 286-295 (2009).
79. Beier, J. *et al.* Collagen matrices from sponge to nano: new perspectives for tissue engineering of skeletal muscle. *BMC Biotechnol* **9**, 1-14 (2009).

80. Ma, J., Holden, K., Zhu, J., Pan, H. & Li, Y. The Application of Three-Dimensional Collagen-Scaffolds Seeded with Myoblasts to Repair Skeletal Muscle Defects. *Journal of Biomedicine and Biotechnology* **2011** (2011).
81. Michael Mitterberger, R.M., Eva Margreiter, Germar M. Pinggera, Daniela Colleselli, Ferdinand Frauscher, Hanno Ulmer, Martin Fussenegger, Georg Bartsch, Hannes Strasser, Autologous myoblasts and fibroblasts for female stress incontinence: a 1-year follow-up in 123 patients. *BJU International* **100**, 1081-1085 (2007).
82. Mitterberger, M. *et al.* Myoblast and Fibroblast Therapy for Post-Prostatectomy Urinary Incontinence: 1-Year Followup of 63 Patients. *The Journal of Urology* **179**, 226-231 (2008).
83. Kirchin, V. *et al.* Urethral injection therapy for urinary incontinence in women. *Cochrane Database of Systematic Reviews* **2** (2009).
84. Siminiak, T. *et al.* Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial. *European Heart Journal* **26**, 1188-1195 (2005).
85. Smits, P.C. *et al.* Catheter-Based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: Clinical experience with Six-Month Follow-Up. *Journal of the American College of Cardiology* **42**, 2063-2069 (2003).
86. Owen, S.C., Li, H., Sanders, W.G., Cheung, A.K. & Terry, C.M. Correlation of tissue drug concentrations with in vivo magnetic resonance images of polymer drug depot around arteriovenous graft. *Journal of Controlled Release* **146**, 23-30 (2010).
87. Burks, S.R. *et al.* Investigation of Cellular and Molecular Responses to Pulsed Focused Ultrasound in a Mouse Model. *PLoS ONE* **6**, e24730 (2011).
88. Chen, X., Cvetkovic, D., Ma, C.-M. & Chen, L. Quantitative study of focused ultrasound enhanced doxorubicin delivery to prostate tumor in vivo with MRI guidance. *Medical Physics* **39**, 2780-2786 (2012).
89. Yang, F.-Y. *et al.* Focused ultrasound and interleukin-4 receptor-targeted liposomal doxorubicin for enhanced targeted drug delivery and antitumor effect in glioblastoma multiforme. *Journal of Controlled Release* **160**, 652-658 (2012).
90. Burgess, A. *et al.* Targeted Delivery of Neural Stem Cells to the Brain Using MRI-Guided Focused Ultrasound to Disrupt the Blood-Brain Barrier. *PLoS ONE* **6**, e27877 (2011).
91. Rogers, M.A. & Evans, W.J. Changes in skeletal muscle with aging: effects of exercise training. *Exercise and sport sciences reviews* **21**, 65-102 (1993).
92. Schultz, E. & Lipton, B.H. Skeletal muscle satellite cells: Changes in proliferation potential as a function of age. *Mechanisms of Ageing and development* **20**, 377-383 (1982).
93. Strasser, H. *et al.* AGE DEPENDENT APOPTOSIS AND LOSS OF RHABDOSPHINCTER CELLS. *The Journal of Urology* **164**, 1781-1785 (2000).
94. Carlson, M.E. & Conboy, I.M. Loss of stem cell regenerative capacity within aged niches. *Aging Cell* **6**, 371-382 (2007).
95. Stölting, M. *et al.* 173 Impact of patient age or gender on bioengineering of functional muscle tissue using muscle precursor cells. *The Journal of Urology* **185**, e72-e72 (2011).
96. Korsgren, O., Andersson, A., Jansson, L. & Sundler, F. Reinnervation of Syngeneic Mouse Pancreatic Islets Transplanted Into Renal Subcapsular Space. *Diabetes* **41**, 130-135 (1992).
97. Sinson, G., Voddi, M. & McIntosh, T.K. Combined fetal neural transplantation and nerve growth factor infusion: effects on neurological outcome following fluid-percussion brain injury in the rat. *Journal of Neurosurgery* **84**, 655-662 (1996).
98. Dawn M. Delo *et al.* Angiogenic gene modification of skeletal muscle cells to compensate for ageing-induced decline in bioengineered functional muscle tissue. *BJU International* **102**, 878-884 (2008).

99. Shefer, G., Rauner, G., Yablonka-Reuveni, Z. & Benayahu, D. Reduced Satellite Cell Numbers and Myogenic Capacity in Aging Can Be Alleviated by Endurance Exercise. *PLoS ONE* **5**, e13307 (2010).
100. Sandri, M.L., Jiandie; Handschin, Christoph; Yang, Wenli; Arany, Zoltan P.; Lecker, Stewart H.; Goldberg, Alfred L.; Spiegelman, Bruce M. PGC-1 alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. *Proceedings of the National Academy of Sciences* **103**, 16260-16265 (2006).
101. Handschin, C. Regulation of skeletal muscle cell plasticity by the peroxisome proliferator-activated receptor γ coactivator 1α. *Journal of Receptors and Signal Transduction* **30**, 376-384 (2010).
102. Arany, Z. *et al.* HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1[agr]. *Nature* **451**, 1008-1012 (2008).
103. Handschin, C. *et al.* PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. *Genes & Development* **21**, 770-783 (2007).

FIGURES

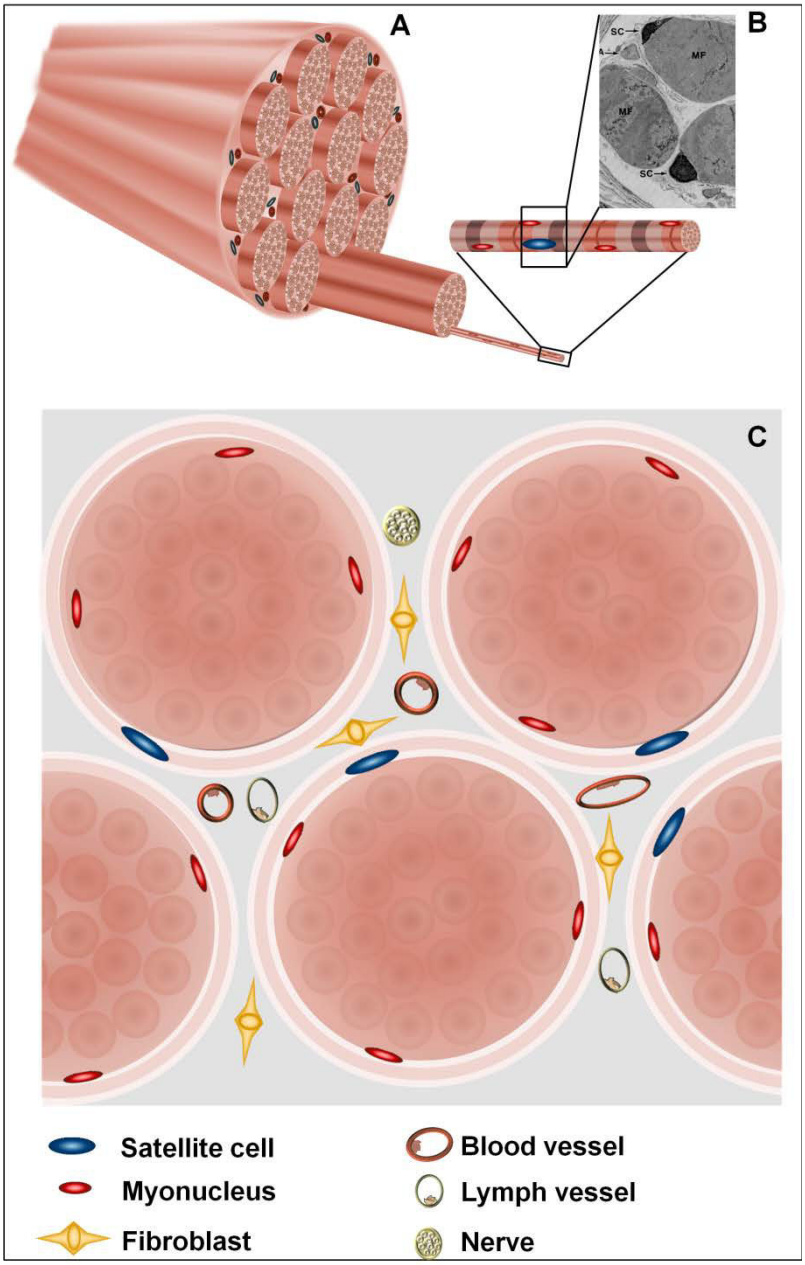

Figure 01 – The muscle niche is the secret of skeletal muscle astounding regenerative capacity. Attached to bones, skeletal muscle are organs composed of skeletal muscle tissue, connective tissue, nerves and blood vessels. Each individual skeletal muscle is composed by hundreds or thousands bundles of muscle fibers that are single cylindrical muscle cells. (A) The connective tissue surrounding each muscle is called epimysium, and its projections that separe muscle bundles are called perimysium. (B) Between single muscle fibers the present connective tissue is called endomysium and it is the muscle satellite cells (SCs) niche. SCs are subsarcolemmal cells that can be activate to regenerate or to compose new muscle fibers. (C) Skeletal Muscle is not only formed by muscle fiber, but also by acellular matrix, cellular components, blood and lymphatic vessels and nerves. Altogether, these muscle niche components play a distinct role on muscle regeneration and on muscle progenitor cell regulation.

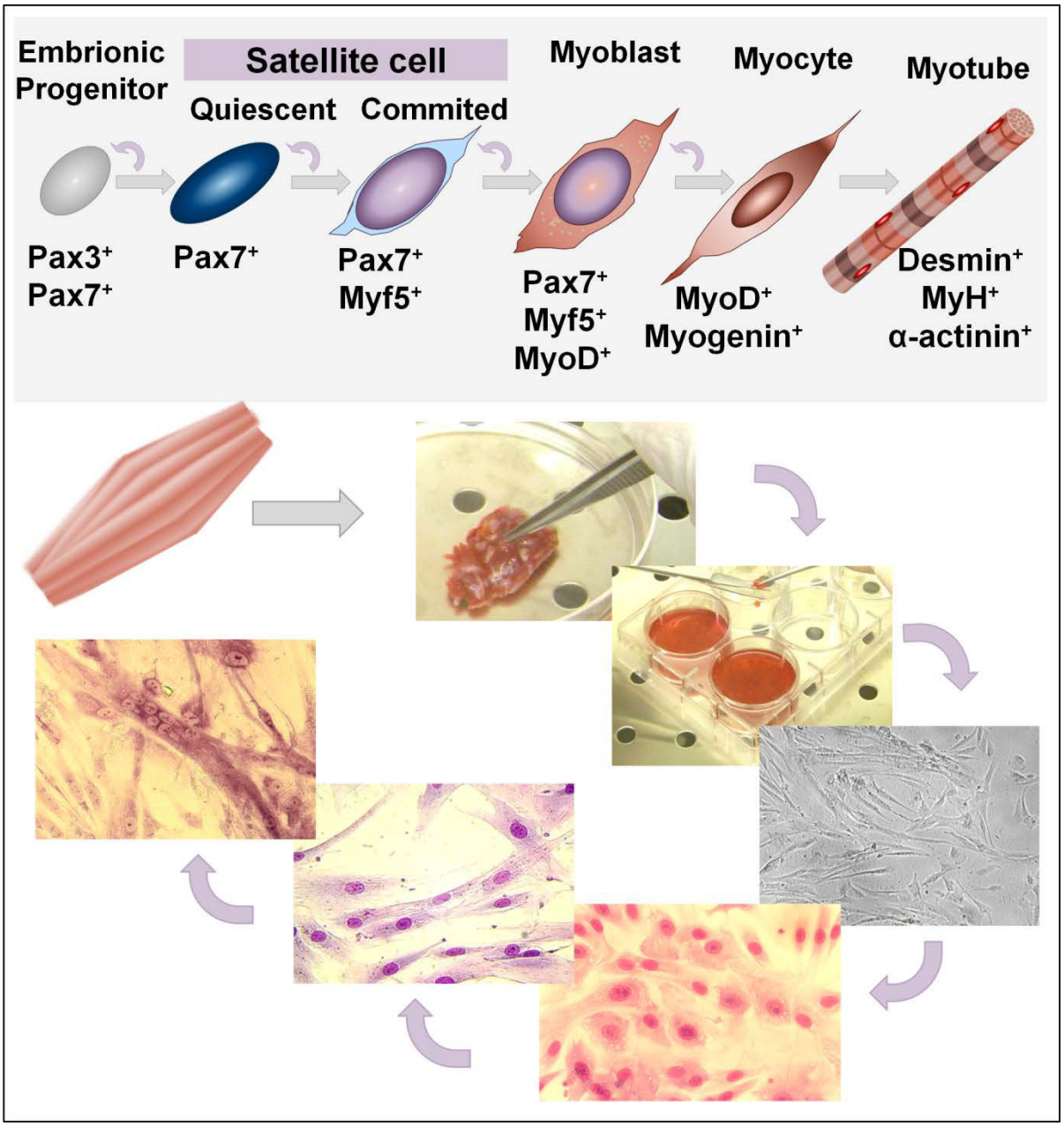

Figure 02 – Myogenic cell characterization and culture. Myogenic cell lineage can be identified in each differentiation state and pursue tightly regulated proliferation and differentiation cycles. From the embryonic state until the terminal differentiation into muscle fibers an intricate network of transcription factors regulates the fate of muscle progenitor cells. These cells can be isolated from any skeletal muscle tissue, grown in culture and reimplanted into a damaged muscle to promote muscle regeneration.

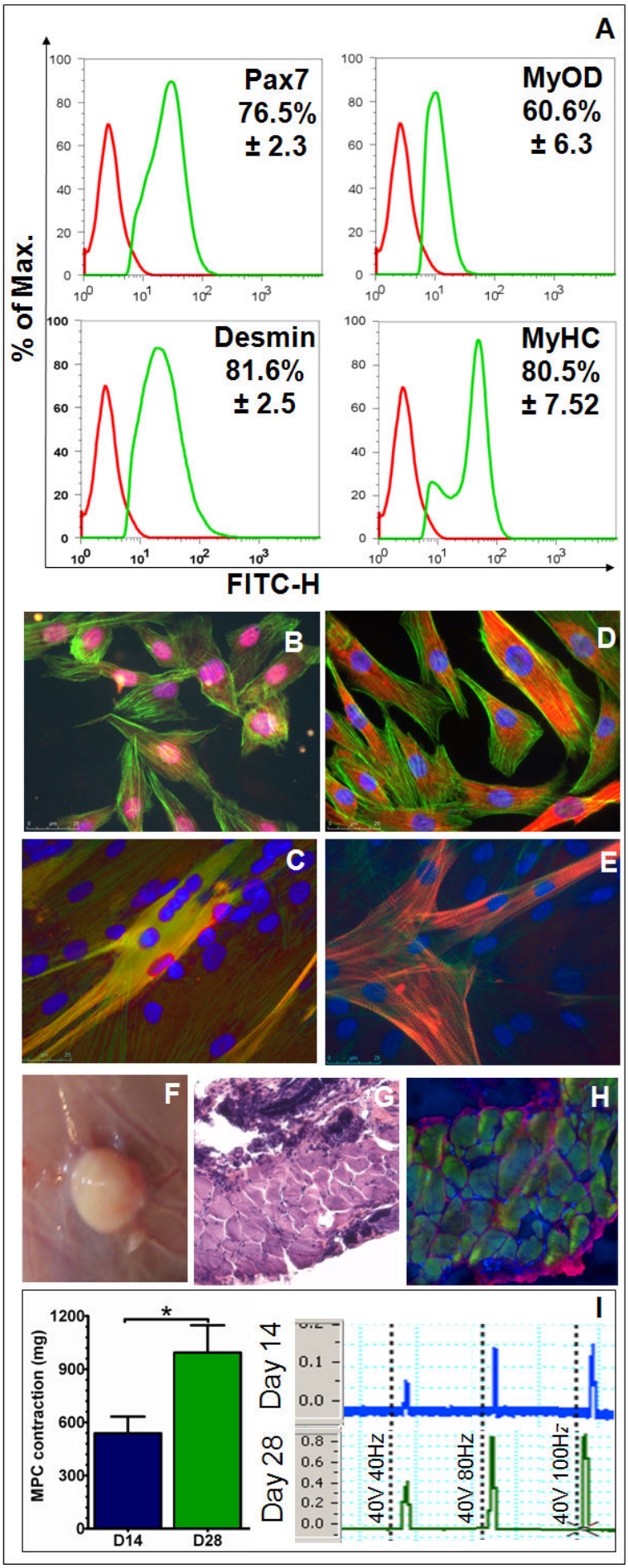

Figure 03 – **Muscle progenitor cells identification *in vitro* and muscle formation after transplant *in vivo*.** Myogenic cells isolated from the *Rectus abdominis* of patients undergoing abdominal surgery, grown in culture and characterized by FACS, immunohistochemistry *in vitro*. Tissue formation was evaluated *in vivo* by Hematoxilin and Eosin staining and immunohistochemistry. Function was assessed by electromyography. A: FACS analyses of cells in P2 expressing Pax 7, MyOD, desmin and upon differentiation induction Myosin Heavy Chain (MyHC). An IgG Isotype control (red curve) was used to determine the background, whereas positive cells are plotted as a green curve. Immunocytochemistry of cells in culture expressing, MyOD (B), MyHC (C), desmin (D), sarcomeric α-actinin (E) (green -Phalloidin 488, blue – DAPI, red - mM anti-IgG Cy3). Muscle cells injected subcutaneously in nude-mice revealed muscle formation *in vivo* (F, G, H) and contraction upon eletrical stimulation (I). HE stained (G) and labelled with sarcomeric α-actinin-Cy3 and PKH67 (H). Muscle function significantly improved over time (I), with contraction strength still increasing after 4 weeks.*p=0.015

CHAPTER 1 – MYOBLASTS INHIBIT PROSTATE CANCER GROWTH BY PARACRINE SECRETION OF TNF ALPHA

Meline N. L. Stölting[1], MD; Stefano Ferrari[2], PhD; Christoph Handschin[3], PhD; Attila Becskei[4], PhD; Maurizio Provenzano[1], MD. PhD; Tullio Sulser[1], Prof.; Daniel Eberli[1]*, MD. PhD

[1]Laboratory for Urologic Tissue Engineering and Stem Cell Therapy, Division of Urology, University of Zurich, Frauenklinikstrasse 10, CH 8091 Zurich, Switzerland; [2]Institute of Molecular Cancer Research, University of Zurich, Winterthurerstr 190, CH-8057 Zürich, Switzerland; [3]Biozentrum, Focal Area Growth and Development, University of Basel, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland; [4]Institute of Molecular Biology, University of Zurich, Winterthurerstr 190, CH-8057 Zürich, Switzerland

* Corresponding Author: Daniel.Eberli@usz.ch, Phone: +41 44 255 9619, Fax: +41 44 255 9620

Accepted at ***Journal of Urology***

ABSTRACT

Purpose: Myoblasts are capable of forming muscle fibers after transplantation and are therefore envisioned as a treatment for urinary incontinence after radical prostatectomy. However, the safety of this treatment and the interaction of myoblasts with remaining neighboring cancer is unknown. We investigated the interactions between myoblasts and prostate carcinoma cells *in vitro* and *in vivo*.

Materials and Methods Myoblasts isolated from *rectus abdominis* were used in a series of co-culture experiments with prostate cancer cells and subcutaneously co-injected *in vivo*. Cell proliferation, cell cycle arrest and apoptosis of cancers in co-culture with myoblasts were assessed. Tumor volume and metastasis formation were evaluated in a mouse model. Tissue specific markers were assessed by immunohistochemistry, FACS analyses, Western blot and RT-qPCR.

Results: In this study we have demonstrated that myoblasts, in proximity of tumor, provide paracrine TNFα to their microenvironment, decreasing tumor growth of all prostate cancer cell lines examined. Co-culture experiments showed induction of cell cycle arrest, tumor death by apoptosis and increased differentiation of myoblasts. This effect was largely blocked by TNFα inhibition. The same outcome was demonstrated in a mouse model, where co-injected human myoblasts also inhibited tumor growth and metastasis formation of all prostate cancer cell lines evaluated.

Conclusions: Myoblasts restrict prostate cancer growth and limit metastasis formation by paracrine TNFα secretion in vitro and in vivo.

Keywords: Prostate cancer, metastasis, autologous transplantation, Anticarcinogenic Agents, TNF-alpha

INTRODUCTION

Skeletal muscle comprises nearly 50% of the human body, is richly vascularized but rarely the site of cancer metastases. The cellular and molecular mechanisms underlying this phenomenon are not yet understood [1]. An intrinsic protective mechanism avoiding ingrowth of metastatic cells and formation of new tumors seems to exist. At the same time, skeletal muscles are the source of myoblasts [2], which are capable of regenerating muscle fibers, and therefore are investigated for the treatment of several muscular dysfunctions, including stress urinary incontinence (SUI) [3], a common complication after radical prostatectomy [4]. Preclinical studies have demonstrated that myoblasts, when implanted in the urinary sphincter, efficiently restore continence [5]**.** The pelvic floor is also a frequent site of residual prostate cancer cells [6], but until now no investigations targeted cell fate and possible interactions between myoblasts and vicinal preexisting cancer.

Parallels have long been drawn between stem cells and cancer cells. In fact, both cell types share common features such as capacity for self-renewal, differentiation potential, relative quiescence, resistance to drug and toxins, resistance to apoptosis, secretion of growth factors and stimulation of angiogenesis by production of vascular endothelial growth factor (VEGF) [7]. These features could result in two possible outcomes: Cell proliferation or cell death. For instance, the presence of VEGF, which is secreted by many stem cells and progenitor cells including myoblasts [8], has the potential to promote prostate cancer angiogenesis leading to enhanced tumor growth and bone metastasis [9]. On the other hand, myoblasts are activated by inflammation and use inflammatory cytokines to perform and regulate their cross-talk for activation and differentiation [2]. These same inflammatory cues, paracrine secreted by myoblasts, could triggers cancer apoptosis.

A recent study successfully demonstrated inhibition of melanoma cell growth in the presence of myoblasts, but failed to describe a possible intercellular mechanism that explains this cell

behavior [10]. Upon differentiation, myoblasts secrete tumor necrosis factor alpha (TNFα), which plays a key role in myoblast activation and differentiation, thereby linking inflammation to muscle regeneration [11]. In tumor cells, TNFα activates two parallel pathways, nuclear-factor-κB (NF-κB) or c-Jun N-terminal kinase (JNK). If NF-κB is activated, TNFα acts as a growth promoter, stimulating proliferation and metastasis. However, if JNK is turned on, a Caspase3-dependent-apoptosis-pathway leads to cell death [12]. In this study, we demonstrate that myoblast-secreted-TNFα is capable of influencing vicinal prostate carcinoma by inducing cell cycle arrest and apoptosis *in vitro* and *in vivo*. Additionally, despite the proximity to prostate cancer, myoblasts will rapidly differentiate into well-organized myotubes. Cell-therapy with myoblasts might provide an ideal treatment of post-prostatectomy urinary incontinence by improving sphincter function and inhibiting potential recurrent prostate cancer growth in the pelvic floor.

MATERIAL AND METHODS

Cell Isolation and Culture

Upon ethical-approval and informed-consent, myoblasts were isolated from *rectus abdominis* biopsies of four male patients (65 ± 6.4y) undergoing abdominal surgery. Biopsies were immediately processed according to established protocols [13] and cells were expanded until passage 2 (P2) with a medium change every third day. Cell characterization was performed by FACS, Immunocytochemistry, RT-qPCR and Western Blot. Muscle tissue formation was assessed by injecting 5 million myoblasts with a collagen carrier (Collagen-type-I Rat tail,BD) into the subcutaneous space of nude-mice. Tissues were retrieved after three and six weeks for histological analysis.

The three prostate carcinoma cell lines (ATCC-LGC Standard) were chosen according to their increasing clinical aggressiveness. They are retrieved from lymph node (LNCaP), bone (PC3), and brain (DU145) metastasis. An aggressive vulvar leiomyossarcoma cell line (SK-LMS1) served as an additional cancer control. An indirect co-culture model (BD Falcon™) was applied, where cells shared culture medium (DMEM enriched with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin) thereby exchanging their cellular products without direct cell-cell-contact. Cancer cells were co-cultured with myoblasts of each patient for 10 day and analyses were performed at days 1, 4, 7 and 10. Myoblasts alone and cancer cell lines cultures were used as control. All experiments were performed in triplicates and medium change was done every third day. TNFα was neutralized with a mouse monoclonal anti-TNFα antibody (Sigma,T-6817).

Growth Rate and Fiber Formation Assay (FFA)

In all cases, triplicate samples of log phase cells were plated at a density of $5x10^3$cells/cm^2.

Cell number and viability was confirmed after trypsinisation by toluidine blue staining. The formation of myofibers was examined on slide chambers and, after 8 days in differentiating condition myofibers were fixed (methanol, 7min), stained (1:20 Giemsa, 1h) and air dried. Images were taken with a Leica-Imager-M1 Microscope. Five high-power-fields (HPF) were analyzed and data expressed as fused cells in myofibers/HPF, number of fibers/HPF and cells per fiber.

Western Blot

Cells were washed with PBS/protease inhibitor (Sigma) and lysed with lysis buffer (50mM Tris-HCl, 150mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 40mM ß-glycerophosphate, 50mM sodium-fluoride, 10µg/ml leupeptin, 10µg/ml aprotinin, 1µM pepstatinA, 1mM PMSF). Samples were centrifuged (10min, 13000rpm), and proteins determined in the supernatant. Culture medium proteins were concentrated by filtering through a 10kDa filter (Amicon Ultra, Millipore). Total protein was measured using DC™ Protein-Assay (Bio-Rad), and 30 µg of protein lysate was loaded on 12% Biorad gels. Proteins were transferred onto PVDF-membranes (Millipore), blocked (1h, 5% non-fat-dry-milk), and incubated (4°C, overnight) with anti-Desmin (1:100,BD Biosciences), anti-MyH (1:6,DSHB), anti-TNFα (1:500,Sigma), anti-$p21^{WAF1}$ (1:1000,Calbiochem), Cleaved Caspase-3-Asp175 (1:1000,Cell- Signaling) and anti-GAPDH (1:2000,Sigma). Membranes were washed, incubated with HRP-conjugated secondary antibody and developed by ECL-technique (ECL-Kit,Amersham).

RT-qPCR

RNA extraction, cDNA preparation and RT-qPCR reactions were done using Taqman® gene expression assay kits (Applied Biosystems), according to manufacturer's protocols. Data were

normalized with 18S expression, quantitatively analyzed by quantification cycles (C_q) and fold changes and graphically represented in amplification plots. MIQE guidelines were followed.

In vivo Experiments and Tumor Size Determination

Myoblasts and tumor cells were cultured as described above, mixed with a collagen carrier (1mg/ml) and bilaterally injected into the dorsal subcutaneous space of 8 nude-mice (n=16 samples) per group. Cell-cell interactions *in vivo* were examined on day 21 and 42 after injection using nine groups: Myoblast ($5x10^6$) alone and co-injected with each of the four cancer cell lines (LNCaP, PC3, DU145 and SK-LMS, $2.5x10^6$) and the four tumors alone ($2.5x10^6$).

The experiments were performed in triplicates and repeated four times with myoblasts from 4 different patients. Tumor volume and growth was measured ($mm^3=a^2xb/2$). Animals were sacrificed by CO_2 asphyxiation. Tumor/sample size, myoblast differentiation, tumor aggressiveness and metastasis (lymph node, lung and liver) were assessed after 21 and 42 days by histological staining.

Histological Staining and Immunocytochemistry

Cells/tissues were fixed (4% PFA), permeabilized (0,5% Triton), blocked (5% BSA/0,1% Triton) and immunolabelled with anti-Pax7 (1:200,Sigma), anti-MyoD (1:100,BDPharmingen), anti-Desmin (1:50,BDBiosciences), anti-MyH (1:4,DSHB) and anti-sarcomeric actinin (1:1000,Sigma), and incubated 1 hour with anti-mouse-IgG-Cy3 (Sigma). Digital images were taken with a Leica Imager M1 Microscope. Tissues were also stained with Hematoxylin and eosin (HE). Histomorphometric analysis was performed using "IMAGEJ for microscopy".

Statistics

Presented data are expressed as averages with corresponding standard deviation. Analyses by independent samples t-tests, one way ANOVA or Pearson Correlation were done with SPSS v20 (SPSS Inc,Chicago,IL). A $p<0.05$ was considered significant.

RESULTS

Cancer cells undergo cell cycle arrest and apoptosis in co-culture with myoblasts *in vitro*

Human myoblasts were successfully isolated and characterized by FACS, Immunocytochemistry (ICC) and Fiber Formation Assay (FFA). The cell population expressed 76.5 ± 2.3% PAX-7, 60.6 ± 6.3% MyOD, and 81.6 ± 2.5% desmin.

After 10 days in co-culture cancer cell lines significantly decreased ($p<0.001$) their growth by 87.9±0.3% for DU145, 66.0±0.1% for LnCAP, 33.6±6.7% for PC3, and 42.0±0.6% for SK-LMS1 (Figure 1A, 1B, 1C). The decrease in cancer cell growth was due to apoptosis and cell cycle arrest, as demonstrated by RT-qPCR and Western Blot (Figure 1D). LnCAP and PC3 showed a >9 fold increase of mRNA for Caspase 3 and $p21^{WAF}$ when grown in co-culture, while DU145 and SK-LMS showed a lower but still significant increase. Western Blot confirmed these data.

Myoblasts in co-culture with cancer cells (Figure 1E, 1F) as well displayed a decreased growth rate (Figure 1G), fused and developed muscle fibers (Figure 1H, $p<0.001$). While only 4.2 ±1.0% of the control myoblasts formed muscle fibers, the differentiation ratio of myoblasts increased in parallel to the respective prostate cancer line aggressiveness ($p=0.011$), with rates of 11.5 ±5.5% in LNCaP, 14.5 ±6.9% in PC3 and 25.3 ±2.3% in DU145.

TNFα-dependent induction of cell cycle arrest and apoptosis in cancer cell lines co-cultured with myoblasts.

Myoblasts demonstrated a striking, up to 25 fold, increase of TNFα mRNA when exposed to tumor (Figure 2A), which lead to significantly higher amounts of TNFα protein ($p<0.001$) into the conditioned-medium in the co-culture system (Figure 2B). The myoblast-TNFα-secretion in co-culture increased gradually according to the corresponding prostate carcinoma

aggressiveness ($p<0.001$), significantly correlating (Pearson:0.754, $p<0.001$) with the myoblast differentiation ratio (Figure 2C). TNFα-antibody blocking in co-culture permitted cancer growth at day 10 to 84.0±0.3% for LnCAP, 99.1±11.4% for PC3, 23.3±0.3% for DU145 and 82.2±1.4% for SK-LMS1 (Figure 2D) of control . Using TNFα blocking in co-culture also decreased the myoblast differentiation ratio by reducing Caspase3 and $p21^{WAF}$ mRNA and protein expression to control-levels (Figure 2E).

The myoblast-secreted-TNFα concentration negatively correlated to cancer cell growth ($p<0.001$, Pearson value -0.58) suggesting that myoblast-paracrine-TNFα is sufficient to induce significant cancer growth inhibition. A parallel assay demonstrated that cancer cell lines alone do not reach detectable levels of TNFα mRNA or protein expression.

Myoblasts restrain tumor growth inducing cancer apoptosis and cell cycle arrest *in vivo*

Interactions between myoblasts and prostate cancer were further investigated *in vivo* by co-injecting myoblasts and tumor cells subcutaneously in nude-mice (Figure 3A). All co-injected samples showed a significantly reduced tumor growth after 21 and 42 days (Figure 3A, 3B. $p<0.05$). Despite rigorous mixing before injection, muscle and cancer grew in distinct clusters of each cell type (Figure 4). Imunohistochemistry was able to confirm the higher level of Caspase 3 and $p21^{WAF}$ expression in all co-injected cancer samples.

Lymph node micrometastasis (Figure 3C) were significantly reduced in co-injected groups (10.9%), when compared to control (90.6%, $p<0.001$). No metastasis to lung and liver were detected. The extent to which myoblasts influenced cancer growth was again proportional to cancer aggressiveness (Figure 4). Histomorphometric distance analyses demonstrated that the tumor areas closer to the newly formed muscle underwent apoptosis and cell cycle arrest more intensely (Pearson: -0.91 and -0.86 respectively) supporting the hypothesis that soluble factors are responsible for the antitumor effects (Figure 5). Despite the evident changes in

tumor behavior, muscle tissue developed a well-organized and differentiated structure *in vivo*. We could not detect any changes in muscle phenotype in the presence of tumor, which also preserved a similar expression of Desmin and $p21^{WAF}$.

DISCUSSION

Cell-cell interactions play a crucial role in tissue formation, regeneration processes and inflammatory reactions. Cellular signaling between neighboring cells is based on two main mechanisms: Growth modulation by endogenous secretion of active compounds and cell competition. These two mechanisms have been well documented in fibroblasts, which are capable of secreting growth factors and other peptides, thus delivering cues to neighboring cells. Fibroblasts isolated from breast tumoral areas are permissive allowing breast cancer metastasis, whereas fibroblast from normal breast tissue restrict tumor growth [14]. Cell competition has also been proposed to regulate early cancer stages, when developing cancer cells overcome genomic constraints[15]. It triggers apoptosis within and around tumors by promoting rivalry between different anaplastic and normal cell lineages [15].

We have demonstrated that myoblasts in co-culture with cancer cells significantly increase TNFα-secretion *in vitro* and *in vivo*. TNFα has been isolated and described 30 years ago, and its clinical application in cancer therapy has been studied ever since [16]. Due to its systemic toxicity, TNFα is clinically only recommended in the treatment of advanced neoplasia, including sarcoma and melanoma, and in advanced cases, when limb amputation would represent the next step[17]. Myoblasts are known to secrete higher TNFα levels when differentiating [18] and this paracrine-secretion evokes microenvironmental changes, which control muscle regeneration by activating Pax7 in quiescent myoblasts and thereby induce differentiation and muscle formation [11].

In our study myoblast-secreted-TNFα levels increase according to tumor aggressiveness, in accordance with previous findings correlating prostate cancer Gleason-score and inflammatory response to endogenous cytokines [19]. The presence of inflammatory factors related to muscle regeneration plays a role in myoblast-secreted-TNFα regulation [11]. This leads to the hypothesis that specific inflammatory cues delivered by the prostate tumor

stimulate neighboring myoblasts to produce higher TNFα levels. A potential pathway is the increase of TACE production due to stress and nutrient shortage, leading to increased release of endogenous TNFα by muscle cells [20].

To investigate the paracrine influences we used a co-culture system where myoblast and cancer cells share the same environment without cell-cell-contact. All cancer cell lines in co-culture showed a significant decrease in growth and increase in apoptosis and cell cycle arrest. This inhibitory effect was almost completely blocked when TNFα was antagonized. In cancer cells TNFα binds to TNFR-1 receptor triggers Caspase-3 activation leading to an apoptotic cascade and cell death [16]. The dual effect of TNFα inducing differentiation in myoblasts and apoptosis in tumors can be explained by two parallel pathways: activation of p38α and c-Jun N-terminal kinase (JNK). Once p38α is activated, Pax7 initiates myogenesis and myoblast differentiation [11] and, by activating the JNK pathway, triggers cancer apoptosis through a Caspase-3-dependent pathway [12]. A further line of action of TNFα in cancer inhibition affects tumor vascularity, probably due to higher response to TNFα in tumoral vessels by receptor up-regulation (TNFR-1) [16].

Our findings demonstrate that co-injected myoblasts are able to limit cancer growth in vivo, again significantly triggering a cell cycle arrest and apoptosis in the cancer tissue. The histomorphometric distance analysis confirmed the role of a soluble factor in hindering tumor growth, supporting that TNFα might be a key player. Further, a significant reduction of lymph node metastasis formation was shown after co-injection, indicating an effect on confining the cancer to the primary site. We anticipate that investigations targeting cancer-mediated-stress factors on muscle cells will be the focus of future efforts towards a better understanding of interactions between cancer and Adult Stem Cells, such as myoblasts.

This research was based on established models for cancer research, however the complex interactions between stromal and tumor cells were not addressed. We used human myoblasts

and cancer cell lines in an animal model. In order to avoid xenograft rejection nude mice with limited immune response were used.

CONCLUSIONS

Myoblasts can be isolated from muscle patients biopsies, rapidly grown in culture, implanted and thereafter form functional muscle within weeks. Our results indicate that differentiating myoblasts secret TNFα inducing apoptosis and cell cycle arrest in Prostate cancer. These characteristics make myoblasts promising cell source for muscle reconstruction, even in the proximity of cancer.

KEY OF DEFINITIONS FOR ABBREVIATIONS

TNFα – Tumor Necrosis Factor alpha
FACS – Fluorescence-activated cell sorting
WB – Western Blot
RT-qPCR – real time quantitative polymerase chain reaction
SUI – stress urinary incontinence
VEGF – vascular endothelial growth factor
NF-κB – nuclear-factor-κB
JNK – c-Jun N-terminal kinase
FBS – fetal bovine serum
FFA - Fiber Formation Assay
HPF – high-power-fields
PFA – Paraformaldehyde
HE – Hematoxylin and eosin
ICC – Immunocytochemistry
$p21^{WAF}$ – Cyclin-dependent kinase inhibitor 1A
TNFR-1 – Tumor Necrosis Factor receptor 1
Pax7 – Paired box protein 7
MyoD – Myogenic Differentiation

REFERENCES

1.Plaza, J. A., Perez-Montiel, D., Mayerson, J., Morrison, C., Suster, S.: Metastases to soft tissue. Cancer, **112:** 193, 2008

2.Gopinath, S. D., Rando, T. A.: Stem Cell Review Series: Aging of the skeletal muscle stem cell niche. Aging Cell, **7:** 590, 2008

3.Carr, L., Steele, D., Steele, S., Wagner, D., Pruchnic, R., Jankowski, R. et al.: 1-year follow-up of autologous muscle-derived stem cell injection pilot study to treat stress urinary incontinence. Int Urogynecol J, **19:** 881, 2008

4.Klingler, H. C., Marberger, M.: Incontinence after radical prostatectomy: surgical treatment options. Curr Opin Urol, **16:** 60, 2006

5.Eberli, D., Aboushwareb, T., Soker, S., Yoo, J. J., Atala, A.: Muscle Precursor Cells for the Restoration of Irreversibly Damaged Sphincter Function. Cell Transplant, 2012

6.Leventis, A. K., Shariat, S. F., Slawin, K. M.: Local Recurrence after Radical Prostatectomy: Correlation of US Features with Prostatic Fossa Biopsy Findings1. Radiology, **219:** 432, 2001

7.Reya, T., Morrison, S. J., Clarke, M. F., Weissman, I. L.: Stem cells, cancer, and cancer stem cells. Nature, **414:** 105, 2001

8.Mac Gabhann, F., Ji, J. W., Popel, A. S.: VEGF gradients, receptor activation, and sprout guidance in resting and exercising skeletal muscle. J Appl Physiol, **102:** 722, 2007

9.Carducci, M. A., Jimeno, A.: Targeting Bone Metastasis in Prostate Cancer with Endothelin Receptor Antagonists. Clin Cancer Res, **12:** 6296s, 2006

10.Parlakian, A., Gomaa, I., Solly, S., Arandel, L., Mahale, A., Born, G. et al.: Skeletal Muscle Phenotypically Converts and Selectively Inhibits Metastatic Cells in Mice. PLoS ONE, **5:** e9299, 2010

11.Palacios, D., Mozzetta, C., Consalvi, S., Caretti, G., Saccone, V., Proserpio, V. et al.: TNF/p38[alpha]/Polycomb Signaling to Pax7 Locus in Satellite Cells Links Inflammation to the Epigenetic Control of Muscle Regeneration. Cell stem cell, **7:** 455, 2010

12.Wang, X., Lin, Y.: Tumor necrosis factor and cancer, buddies or foes[quest]. Acta Pharmacol Sin, **29:** 1275, 2008

13.Eberli, D., Soker, S., Atala, A., Yoo, J. J.: Optimization of human skeletal muscle precursor cell culture and myofiber formation in vitro. Methods, **47:** 98, 2009

14.Dong-LeBourhis, X., Berthois, Y., Millot, G., Degeorges, A., Sylvi, M., Martin, P. et al.: Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. Int J Cancer, **71:** 42 1997

15. Moreno, E.: Is cell competition relevant to cancer? Nat Rev Cancer, **8:** 141, 2008

16.van Horssen, R., ten Hagen, T. L. M., Eggermont, A. M. M.: TNFalpha in Cancer Treatment: Molecular Insights, Antitumor Effects, and Clinical Utility. Oncologist, **11:** 397, 2006

17.Taeger, G., Grabellus, F., Podleska, L. E., Müller, S., Ruchholtz, S.: Effectiveness of regional chemotherapy with TNF-alpha/Melphalan in advanced soft tissue sarcoma of the extremities. Int J Hyperthermia, **24:** 193, 2008

18.Chen, S.-E., Gerken, E., Zhang, Y., Zhan, M., Mohan, R. K., Li, A. S. et al.: Role of TNFalpha signaling in regeneration of cardiotoxin-injured muscle. Am J Physiol Cell Physiol, **289:** C1179, 2005

19.Nuñez, C., Cansino, J. R., Bethencourt, F., Pérez-Utrilla, M., Fraile, B., Martínez-Onsurbe, P. et al.: TNF/IL-1/NIK/NF-kappaB transduction pathway: a comparative study in normal and pathological human prostate (benign hyperplasia and carcinoma). Histopathology, **53:** 166, 2008

20.Zhan, M., Jin, B., Chen, S.-E., Reecy, J. M., Li, Y.-P.: TACE release of TNFalpha mediates mechanotransduction-induced activation of p38 MAPK and myogenesis. J Cell Sci, **120:** 692, 2007

FIGURES

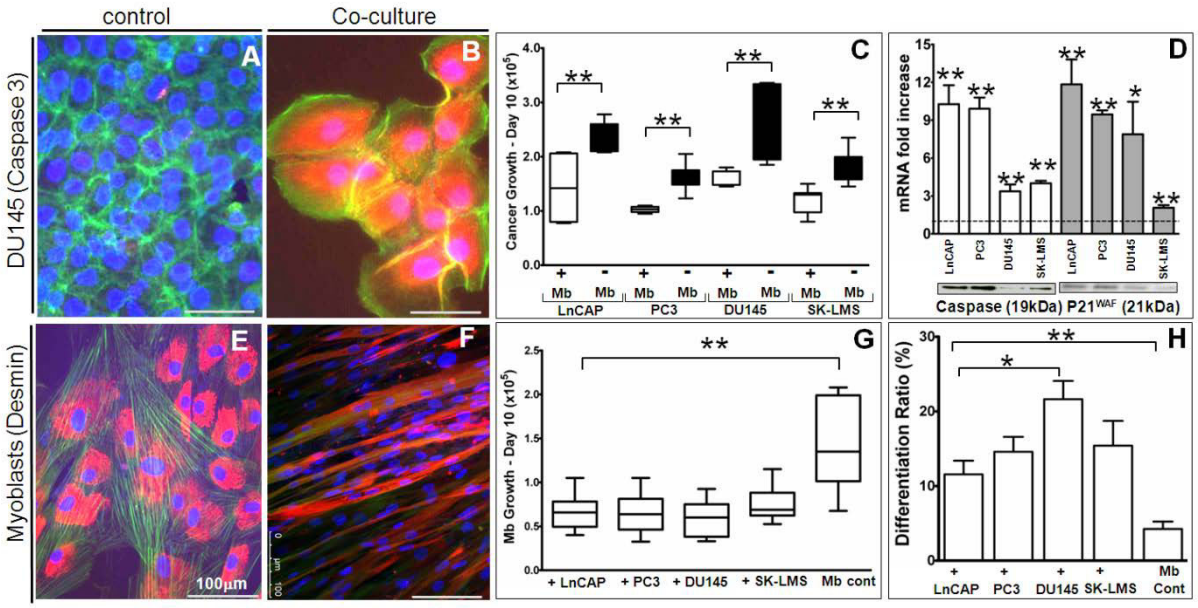

Figure 1 – **Co-culture effects on myoblasts and cancer cells.** Cell growth rate, differentiation ratio, morphology and gene expression were influenced by co-culture. Prostate carcinoma and sarcoma cells significantly decreased in growth (A, B, C) and underwent apoptosis and/or cell cycle arrest (D). Myoblasts differentiated rapidly in the presence of tumor, significantly increasing differentiation ratio (E, F, H) and, consequently, decreasing cell growth (G). Desmin staining in co-culture (A) and control (B), Caspase 3 staining of DU145 cells in co-culture with myoblasts (C) and control (D), cytoskeleton labelled in green (Phalloidin 488) and secondary antibody in red (Cy3). Caspase 3 and p21 mRNA fold increase and protein expression (H) significantly increased when compared to tumor control (dashed line=1.0). Samples in co-culture with myoblast were represented as (+ Mb) and control without myoblasts as (- Mb). mRNA fold increase was normalized with 18S reference gene (*p<0.001, **p=0.005, ***p=0.011)

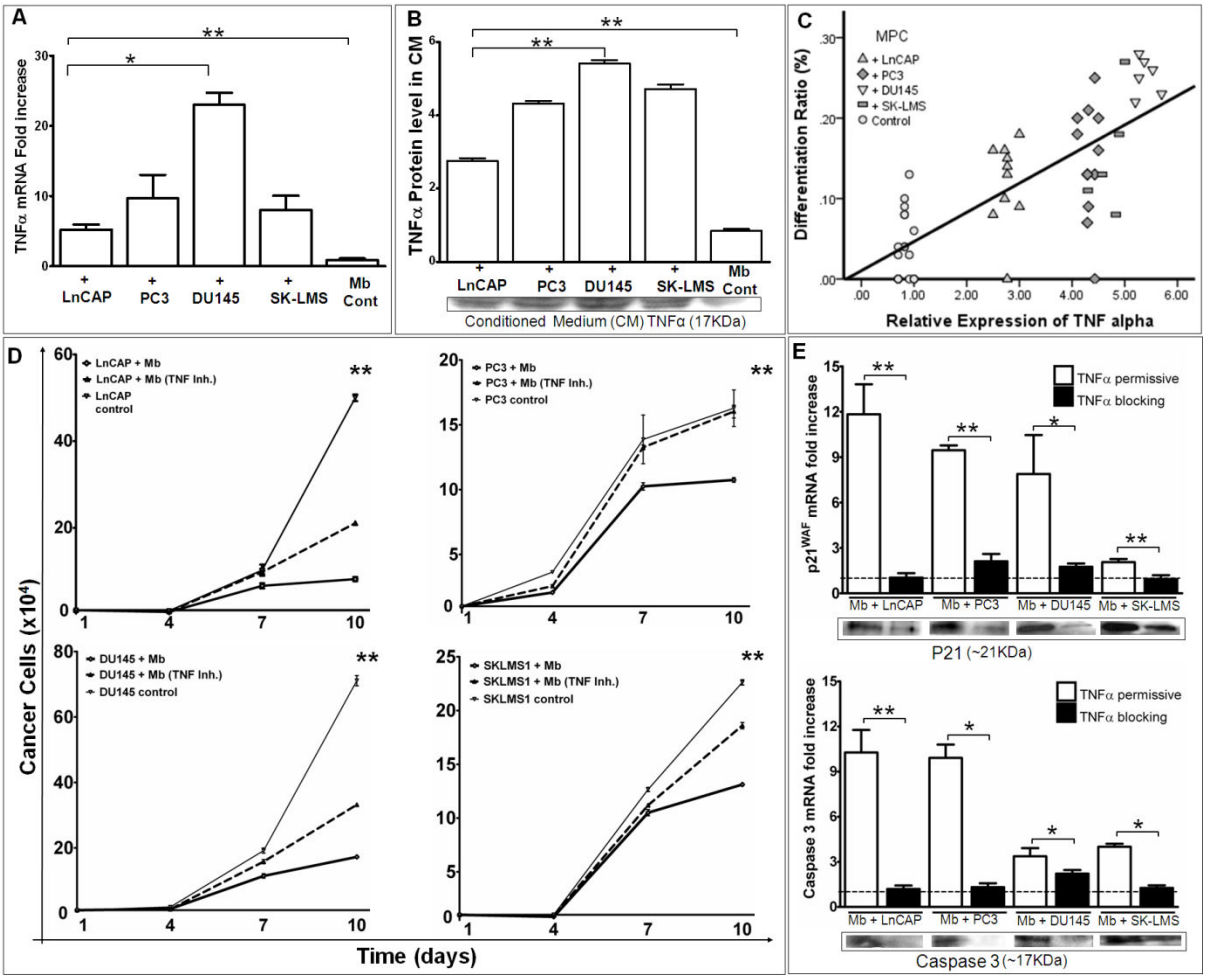

Figure 2 – **Myoblast secreted TNFα induce myoblast differentiation and inhibit cancer cell line growth rate by inducing apoptosis and cell cycle arrest.** (A) RT-qPCR assay demonstrates myoblast **TNFα** mRNA expression is increased after 4 day of co-culture with different cancer cell lines. A significant difference could be found between different prostate cancer cell lines, increasing according to tumor aggressiveness. (B) Myoblasts TNFα secretion increases according to the cancer aggressiveness in co-culture. CM: conditioned Medium. (C) Myoblast differentiation ratio correlate (Pearson correlation: 0.754) to the amount of produced TNFα . (D) Cell growth rate of cancer (LNCaP, PC3, DU145 and SK-LMS-1) was assessed by cell counting at day 1, 4, 7 and 10 of co-culture. All cancer cell lines showed a significant decrease in growth in the presence of myoblasts (bold lines), when compared to control (fine line). Cancer growth rate was in great part recovered (dashed lines) after TNFα neutralization. (E) Apoptosis and cell cycle arrest are triggered in all cancer cells co-cultured with myoblasts. Again TNFα blocking reversed in great part these effects. Caspase 3 and p21 tumor control mRNA fold increase is represented with a dashed line (=1.0). (*p<0.001, **p<0.05).

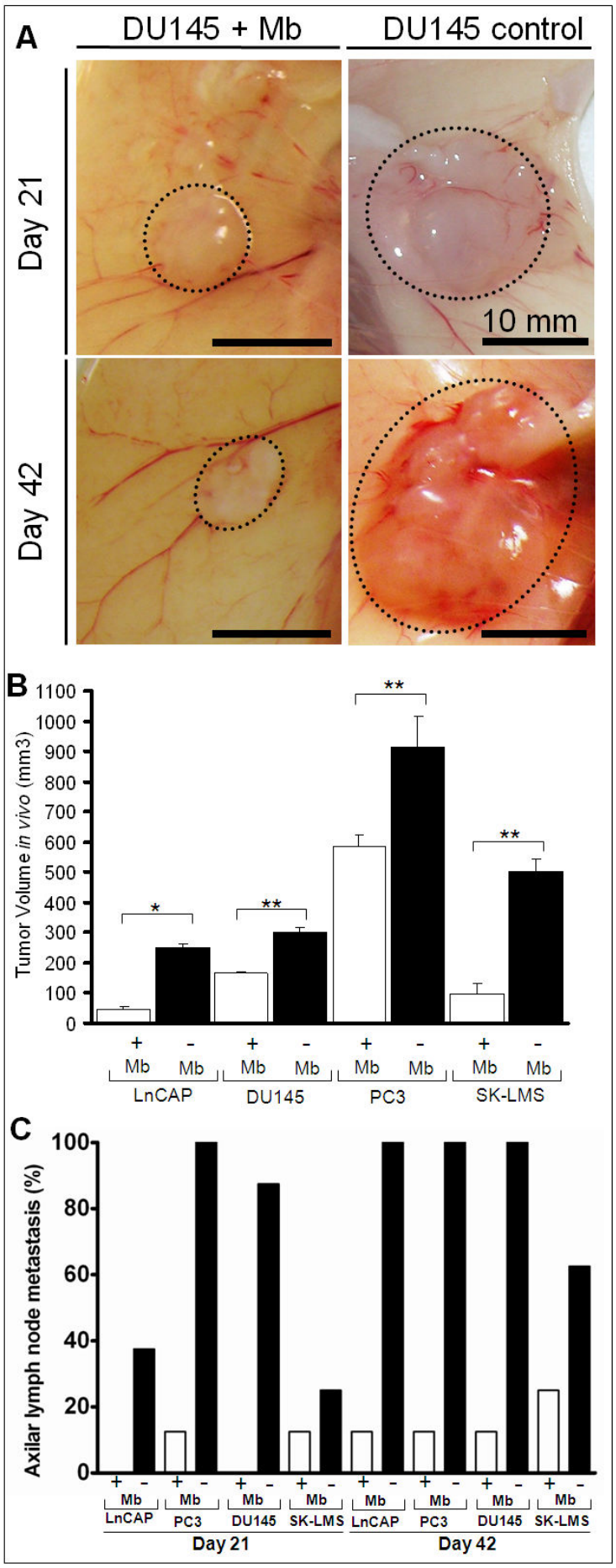

Figure 3 – **Tumor growth and lymph node metastasis was reduced *in vivo* in samples co-injected with myoblasts.** (A) 21 days after subcutaneous cell injection, tumor size was measured and a significant tumor size difference was found between co-injected and control samples. On day 42, myoblast co-injected tumor mass shrank, whereas control samples kept growing. (B) Final tumor size at day 42 was significantly smaller in myoblast co-injected samples. (C) Axillar lymph node metastasis assessment was performed by analysis of metastasis with H&E, Desmin and cytokeratin staining, positive lymph nodes. Ratio of axillar metastasis was also significantly reduced ($p<0.001$) in all tested cancers, when co-injected with myoblasts. Samples co-injected with myoblasts were represented as (+ Mb) and control without myoblasts as (- Mb). *$p<0.05$, **$p=0.009$, ***$p=0.002$

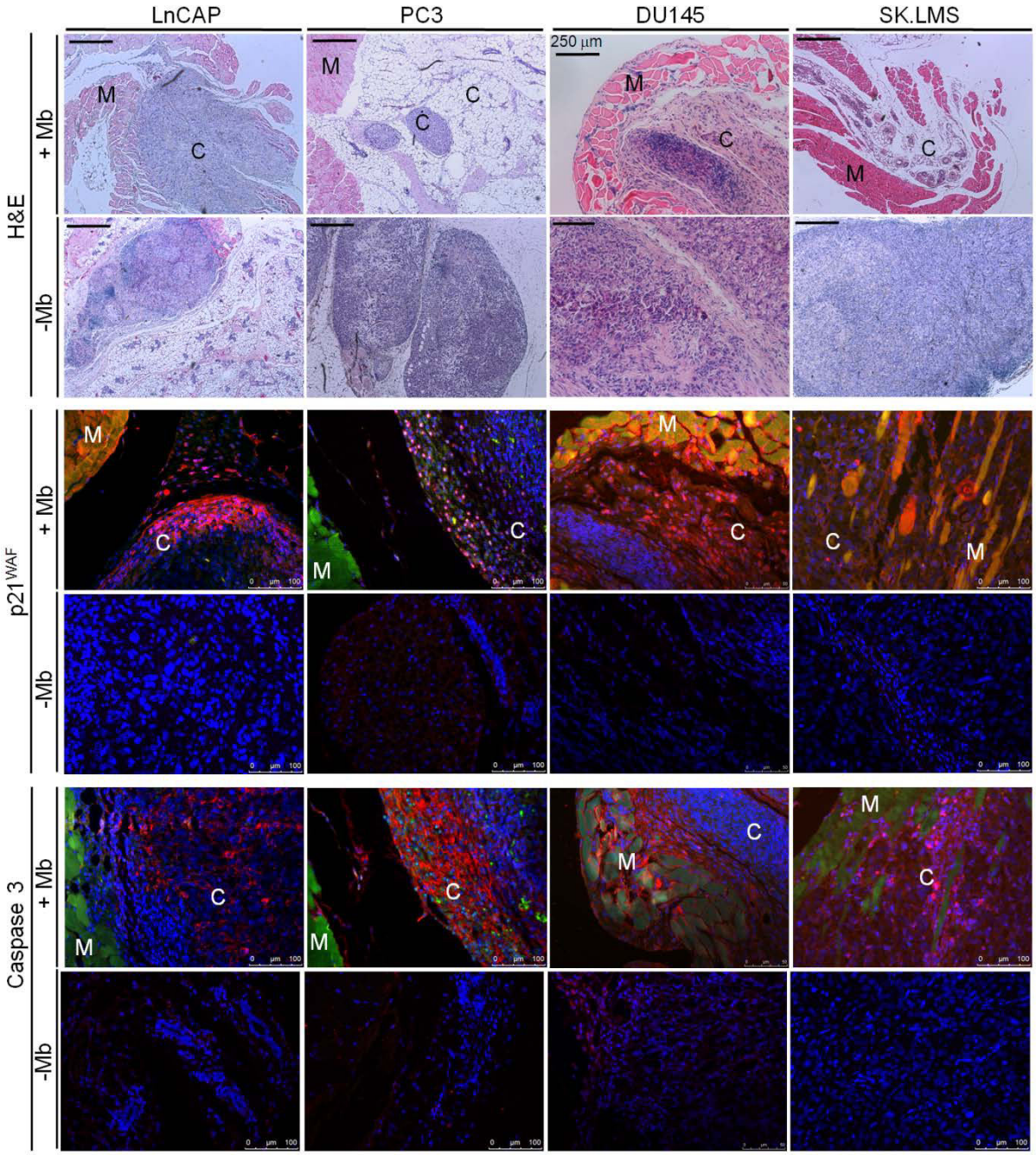

Figure 04 – **Histological analysis of tumor formation *in vivo*.** At day 42, HE staining demonstrates a tendency of newly formed muscle and cancer tissue (first row) to growth in clusters, with differentiated muscle areas impairing growth of neighbor tumor masses. In opposition, control cancers (second row) grow freely forming bigger and complex tumor masses. Increasing Caspase3 and $p21^{WAF}$ expression was detected in all tumors in samples co-injected with myoblasts (third and fifth row). Co-injected myoblasts differentiated into $p21^{WAF}$ positive muscle fibers. Samples co-injected with myoblasts were represented as (+ Mb) and controls are cancer cell lines injected without myoblasts (-Mb). In the co-injected samples muscle is represented with a "M" and cancer tissue areas with a "C". DAPI (blue), anti-mouse IgG Cy3 (red), and the injected myoblasts were labeled *in vitro* with PKH 67 (green).

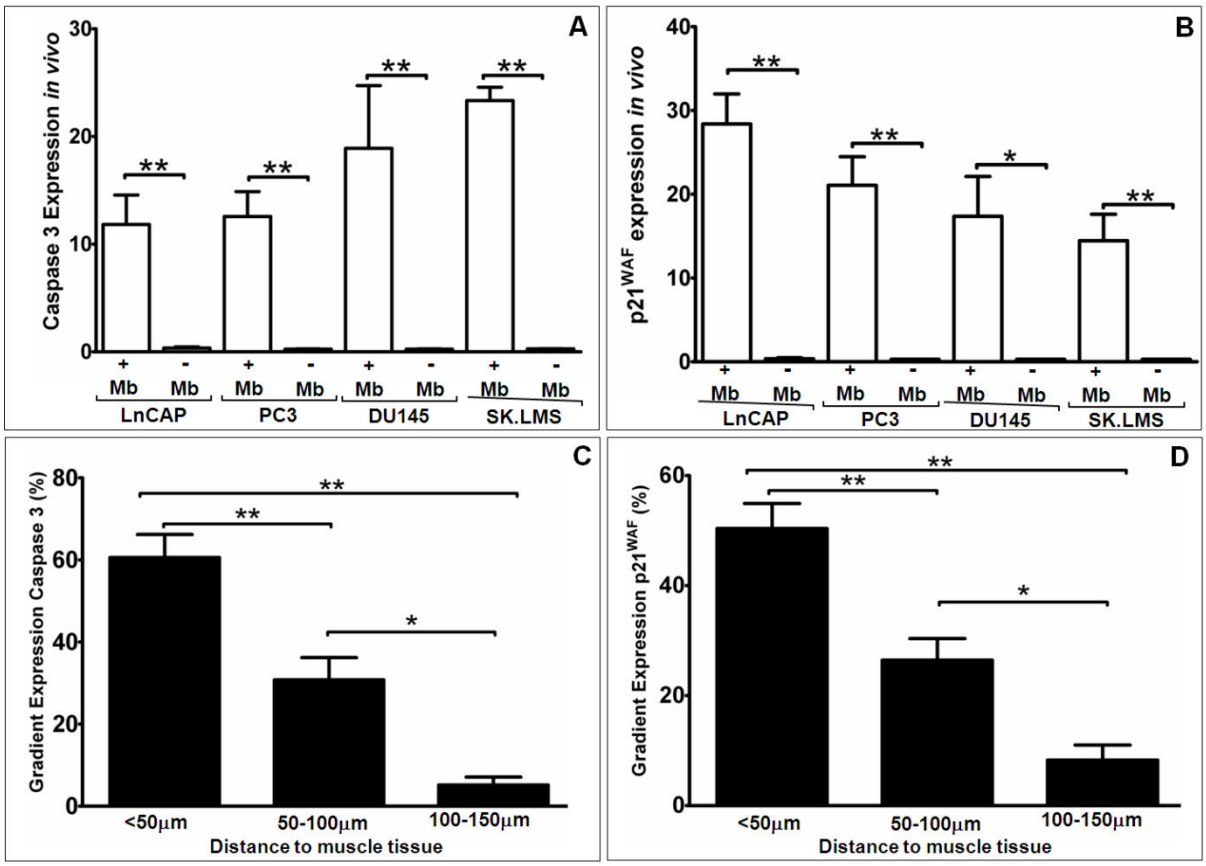

Figure 05 – **Histomorphometric analyses of co-injected tumor with myoblasts.** Histomorphometric analysis demonstrates a significant increase on apoptosis (A) and cell cycle arrest (B) in all co-injected tumors. (C) Three tumor areas were analyzed according to distance of newly formed muscle. The total positive area, calculated by fluorescence intensity, demonstrated a gradient of Caspase 3 and p21 expression in all cancers tested. These directly correlated with the proximity to differentiating muscle tissue. Samples co-injected with myoblast were represented as (+ Mb) and control without myoblasts as (- Mb). *p<0.001, ** p< 0.05, ***p=0.001

The role of donor age and gender on the success of human muscle precursor cell transplantation

Meline N L Stölting[1], Lukas J Hefermehl[1], Mathias Tremp[1], Fahd Azzabi[1], Tullio Sulser[1], Daniel Eberli[1*]

Name of institutions: [1]Laboratory for Urologic Tissue Engineering and Stem Cell Therapy, Division of Urology, University of Zurich, Frauenklinikstrasse 10, CH 8091 Zurich, Switzerland;

* Corresponding Author: Daniel.Eberli@usz.ch, Phone: +41 44 255 9619, Fax: +41 44 255 9620

To Submit to: Regenerative Medicine

Keywords: Age, Gender, Muscle reconstruction, Autologous transplant, Clinical application.

Word count (Abstract): 202

Word count (text): 4300

Abstract

Autologous cell transplantation for the treatment of muscle damage or insufficiency is feasible by applying muscle precursor cells (MPCs) isolated from adult skeletal muscle. At the onset of trauma these cells are recruited to proliferate and rebuild injured muscle fibers. However, a variety of donor specific cues may directly influence the yield and quality of cells isolated from a muscle biopsy. In this study, we isolated human MPCs and assessed the role of donor gender and age on the ability of these MPCs to form functional bioengineered muscle. We analyzed cell yield, growth and molecular expression *in vitro* as well as engraft survival and contractility *in vivo* of samples isolated from men and women in three different age groups: young (20-39y), adult (40-59y) and elderly (60-80y). Our results suggest that although human MPCs can be successfully isolated and grown from patients of all ages and both gender, young and female donors provide fast growing cells *in vitro* with an optimum contractile output *in vivo* and are therefore an ideal cell source for muscle reconstruction. Taken together these findings describe the donor related limitation of MPC transplantation and give insight to a straightforward and unbiased clinical application of these cells for muscle reconstruction.

Keywords: Age, Gender, Muscle reconstruction, Autologous transplant, Clinical application.

Introduction

Tissue regeneration is a biological process in multi-cellular organisms that is essential for survival and adaptation to trauma. This process occurs in most vital organs and is mainly mediated by the activation and differentiation of tissue-specific resident adult stem cells or precursor cells [1]. The depletion and malfunction of these cells become evident in the elderly where a decline in organ maintenance and regenerative potential leads to longer healing periods for wounds and bone fractures, a decrease in skin elasticity and an increased propensity for infections and cancer [2-4]. In skeletal muscle tissue, a specialized precursor cell population with the capability for muscle fiber reconstruction is activated by injury [5]. Muscle Precursor Cells (MPCs) or muscle satellite cells are small dormant progenitor cells, which reside between lamina propria and sarcolemma of single muscle fibers. In the event of muscle trauma, triggered by nitric oxide [6] and TNFα [7], MPCs re-enter the cell cycle, amplify and follow the myogenic differentiation, allowing normal muscle regeneration [8] and muscle turn-over induced in response to exercise[9].

Their easy accessibility through a biopsy and good expansion potential make them excellent candidates for the treatment of a variety of muscle diseases [10]. However, the quality of cells for transplantation is a widely discussed matter. Several variables, including species, age and gender of both the donor and host, may directly influence the ability of the transplanted MPCs to form functional bioengineered muscle. Many of the muscle diseases where MPC-transplantation is envisioned are found within the aged population. Therefore, the age of the donor and recipient should be considered when planning cell transplantation. Recent studies have demonstrated that adult stem cells isolated from old donors present diminished telomerase activity, express lower levels

of growth factor receptors (VEGF, IGF, EGF and G-CSF), contain more DNA double-strand breaks and respond poorly to therapy with antioxidant agents [11,12]. The density of MPCs in adult muscle decline with age due to apoptosis [13]. In fact, the number of MPCs gained from a muscle biopsy and their proliferation potential in culture gradually decline in an age-dependent manner [14]. Additionally, the recipient age seems also to have an influence, as the stem cell regenerative capacity reduces in aged niches [15]. Hence, these cells are an ideal candidate for the treatment of muscle deficits by autologous transplantation, which avoids engraft rejection complications but challenges clinical application in the elderly.

Dimorphic differences in muscle features suggest that donor gender may also play an important role in the quality of precursor cells used for transplantation. Sex-related differences in muscle become evident when male and female total muscle mass is compared. Although muscle mass is clearly higher in men, age-related skeletal muscle mass reduction occurs twice as fast in men than women [16]. Female musculature is more insulin sensitive [17], displays a higher sympathetic nerve activity and norepinephrine sensitivity [18] and is less susceptible to oxidative stress [19]. These gender-dependent metabolic differences might all have a substantial influence on the ability of MPCs to form functional muscle fibers.

In this study, we investigated the impact of donor age and gender on the success of muscle cell transplantation by analyzing samples from men and women in three different age groups: young (20-39 y), adult (40-59 y) and elderly (60-80 y). We evaluated yield of cells, expansion potential, phenotypic characteristics, and survival as well as – after cellular transplant – tissue formation, function and contractile response to tetanic stimulation. This study not only establishes the efficacy of MPC transplantation in rebuilding functional muscle, but more importantly, it defines cell

donor-specific differences as well as pitfalls and benefits that need to be considered before planning clinical application. This is of key importance since many treatments are envisioned for a defined patient population (e.g. sphincter muscle reconstruction in elderly woman). Taken together, these findings are key to a straightforward and unbiased clinical recommendation and application of muscle precursor cell transplantation for striated muscle reconstruction.

Materials and Methods

Subjects

With ethical approval and informed consent, human muscle biopsy samples were obtained from the *rectus abdominis* of 41 hospitalized patients who had undergone abdominal surgery. Only patients without diseases affecting muscle tissue were included in this study. Overall, 23 samples were taken from men and 18 were from women. The samples were divided in donor age groups: 21-40, 41-60 and 61-80 years of age. The weight and size of all biopsies were recorded to calculate the MPC yield.

Cell isolation and culture

Cell isolation and culture were performed as previously reported [15]. Briefly, biopsies were transported in PBS and immediately processed. The remaining conjunctive tissue was micro-surgically removed, and the muscle was minced and digested in a collagenase/dispase (0.4%/0.2%) solution [1 h, 37ºC]. Sample digestion was blocked with DMEM/F12 enriched with 10% FBS and 1% penicillin/streptomycin, centrifuged [1500 rpm, 5 min] and homogenized in DMEM/F12 (Gibco, Grand Island, NY) with 1% penicillin/streptomycin (Gibco), 18% fetal bovine serum (Gibco), 10 ng/ml hEGF (Sigma), 1 ng/ml hbFGF (Sigma), 10 µg/ml human insulin (Sigma) and 0.4 µg/ml dexamethasone (Sigma). Digested muscle fibers were filtered through a 100-µm strainer and plated in 6-well collagen-coated (Collagen-type-I Rat tail,BD) dishes. The purity of the MPCs was improved by allowing the digested muscle fibers to settle for 24 h and replating the non-adhering cells to a new collagen-coated dish. Fast adhering fibroblasts new discharged. The

muscle cells were expanded in growth medium until passage 2 (P2) with a medium change every third day.

Cell growth rate

Growth curves were determined using 24-well plates seeded with MPCs at a density of 5000 cells/cm^2 with cells at passage 1 (P1). At each time point, cells were trypsinized and counted (n=52) with hemocytometer in triplicates. The counts were then averaged and plotted as total cell count vs. time (days). The cell cycle rates were calculated from the average doubling times at the midpoint of the growth curves for each cell type. Cell growth was assessed daily for one week, and cell viability was confirmed by toluidine blue staining and expressed a percent live cells.

Fiber formation and Fusion Rates

For the fiber formation assay (FFA) 5000 cells/cm^2 were plated on slide chambers with serum-reduced medium (DMEM/F12, 10% FBS, 1% penicillin/streptomycin) to induce the formation of myofibers [10]. Muscle cells were kept in this condition for 8 days with a medium change every third day. Slides were then fixed in 100% methanol [7 min], stained with 1:20 Giemsa (Sigma-Aldrich) for 1h, washed with deionized water, and air dried. Images were captured with a Leica Imager M1 Microscope. Differentiation rate was calculated by analysing in 10 high-power-fields (HPF) per sample the number of nuclei in differentiated myofibers/HPF divided by the total number of nuclei/HPF.

Cell characterization by FACS analysis and immunostaining

For FACS, cells immunolabeled with anti-Pax7 (1:100, Sigma), anti-MyoD (1:100,

BD Pharmingen), anti-Desmin (BD 1:50, Biosciences), anti-myosin heavy chain (1:4, DSHB, Yowa), anti-myosin heavy chain type 1 (1:2, DSHB, Yowa), anti-myosin heavy chain type 2 (1:4, DSHB, Yowa) and anti-sarcomeric actinin (1:500, Sigma) were incubated overnight at 4ºC, washed with PBS and incubated with 2 ng/µl FITC goat anti-mouse IgG/IgM antibody (BD Biosciences) for 1 hour at room temperature. A total of 50,000 events were registered immediately after labeling with a Becton Dickinson FACS Canto flow cytometer (BD Biosciences, San Jose, California), and the data were analyzed using FlowJo software v. 7.2.5 (Tree Star Inc., Ashland, Oregon). All data are expressed as the percent of positive cells as defined by flow cytometry.

For immunostaining, cells cultured on slide chambers were fixed in 4% paraformaldehyde for 10 minutes at room temperature, permeabilized in 0.5% Triton for 7 minutes, blocked (1% BSA, 0.1% Triton in PBS) at room temperature for 30 min. Cells were immunolabeled with anti-Desmin (1:100, BD Biosciences), anti-myosin heavy chain (1:2, DSHB, Yowa), anti-myosin heavy chain slow twitch (1:5, DSHB, Yowa), anti-myosin heavy fast twitch (1:2, DSHB, Iowa), anti-MyoD (1:100, BD Pharmingen), anti-sarcomeric actinin (1:500, Sigma) and anti-Pax7 (1:200, Sigma) followed by a Cy3 secondary antibody (1:1000, Sigma). The cytoskeleton was stained with Alexa-Fluor-488-phalloidin (Sigma), and the nuclei were stained with DAPI (Sigma).

Muscle Cell Transplantation and Histology

Ten million cells mixed with a collagen carrier (1 mg/ml, Collagen-type-I Rat tail, BD) were bilaterally injected into the dorsal subcutaneous space of nude mice as previously reported[20]. To rule out an influence of the recipient microenvironment,

all animals were 3 month old female. After 2 and 4 weeks, muscle tissue engrafts were retrieved, weighed, embedded in OCT, and 10-µm frozen sections were cut and air dried. The hematoxylin/eosin staining was performed as described[21] and tissues were also fixed with pre-cooled 100% methanol and immunostained as described above. The antibodies used were anti-Desmin (1:50, BD Biosciences), anti-MyH (1:2, DSHB), anti- MyH1 (1:5, DSHB), anti-MyH2 (1:2, DSHB) and anti-sarcomeric actinin (1:500, Sigma) followed by a Cy3 secondary antibody (Sigma). Images were acquired at exposures that were based on unstained controls with a Leica Imager M1 Microscope (Carl Zeiss, Thornwood, NY). Histomorphometric analysis was performed using the MBF software "IMAGEJ for microscopy".

Western Blot analysis

Western blot analysis was performed as previously described [15]. Briefly, cells were washed with PBS supplemented with a protease inhibitor cocktail (Sigma) and lysed with modified lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 40 mM ß-glycerophosphate, 50 mM sodium fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µM pepstatin A, and 1 mM PMSF). Samples were centrifuged for 10 min at 13,000 rpm, and the supernatant was saved for protein analysis. Total protein was measured using the DC™ Protein Assay (Bio-Rad), and 30 µg of protein lysate were loaded on 12% BioRad gels. Proteins were transferred onto PVDF membranes (Immobilion-P; Millipore, Bedford, MA), blocked for 1 hour in 5% non-fat dry milk, and incubated with primary antibodies at 4 °C overnight. The primary antibodies were anti-Desmin at a dilution of 1:100 (BD Biosciences), anti-myosin heavy chain at 1:6 (DSHB, Yowa), anti-TNFα at 1:500 (Sigma), anti-$p21^{WAF1}$ at 1:1000 (Calbiochem), cleaved caspase-3 (Asp175) at 1:1000

(Cell Signaling Technology) and 1:2000 monoclonal anti-GAPDH (Sigma). Membranes were washed in TBS with 0.1% Tween-20 for 30 min and incubated with the appropriate HRP-conjugated secondary antibody (Amersham, Dübendorf, Switzerland) in TBS with 0.1% Tween-20 and 5% non-fat dry milk for 1 h. The membranes were developed using ECL (ECL-Kit, Amersham, Freiburg, Germany). Tissue protein extraction followed the same protocol, but started with freezing of the samples in liquid nitrogen. The samples were then crushed and ground using a mortar and pestle and lysed with the lysis buffer described above. Protein values were quantified using the NIH software "Image J" (NIH, Bethesda, MD) and normalized against GAPDH.

Organ Bath

After harvest tissue was kept under tension with constant oxygenation (95% O_2 and 5% CO_2) in Krebs solution at 25ºC. Muscle strips (around 5 x 2mm) were fastened with vicryl into the myograph-chambers (DMT, Denmark) and aloud to equilibrate under 20mN (2.0g) for 20 min, adjusting the tension periodically and replacing Kreb's solution every 5-10 min. Single 80V/80Hz twitch stimulations were used to determine the optimum length (L0) of each tissue. Tissue was stimulated by increasing (40, 80, 100Hz at 40V, 80V and 100V consecutively) electrical-field stimulation (EFS). Extensor-digitorum-longus muscle was used as controls. The maximum tension under titanic contractions was registered, normalized to the sample weight (mg/mg tissue). All data was collected using a LabChart v7.0 (ADinstruments, Spechbach, Germany) and expressed as mean/SD.

Statistics

All presented data are expressed as averages with their corresponding standard deviation. SPSS v11 (SPSS Inc., Chicago, IL) was used for statistical analysis, and graphics were drawn with GraphPad Prism v5.04 (GraphPad Software, Inc.). All data were analyzed by unpaired t-tests or one-way ANOVA using Bonferroni post-hoc analysis. $p<0.05$ was considered significant.

Results

Age and gender directly influence cell behavior *in vitro* – growth rate and cell yield are reduced in cells from male and elderly donors

To assess the growth rate of MPCs *in vitro* we have isolated and grown cells from the *rectus abdominis* of individuals undergoing abdominal surgery. The retrieval of muscle biopsies was uneventful and their average weight was 966.4±132.7mg.

Donor gender and age caused growth rate in passage 1 to nearly double at day 4 for female samples (female $4.06\pm0.44x10^6$ and male 2.43 ± 0.24 $x10^6$, $p<0.05$, figure 1A) and young donors (21-40y $4.36\pm0.51x10^6$, 41-60y $2.83\pm0.22x10^6$ and 61-80y $1.88\pm0.20x10^6$ cells, $p<0.05$, figure 1B). Gender isolated analyses of the impact of aging on cell growth demonstrated that cells isolated from young female donors will grow up to 4-fold more than from elderly females (21-40y $8.26\pm2.59x10^6$, 41-60y $3.79\pm1.26x10^6$ and 61-80y $2.26\pm0.69x10^6$ cells, $p<0.001$, figure 1C), while in the male cells the specific effect of aging was milder (21-40y $3.26\pm1.05x10^6$, 41-60y $2.72\pm0.80x10^6$ and 61-80y $2.35\pm0.82x10^6$ cells, $p<0.05$, figure 1D). Accordingly, the total number of precursor cells recovered per gram of tissue more than doubled in female samples (female $11.07\pm1.31x10^5$, and male $4.02\pm0.38x10^5$ cells/g of muscle, $p<0.001$, figure 1E) but decreased with age (21-40y $7.25\pm1.16x10^5$, 41-60y $3.20\pm0.20x10^5$ and 61-80y $2.76\pm0.38x10^5$ cells/g of muscle tissue, $p<0.001$, figure 1F).

The ability to remain in an undifferentiated proliferative state *in vitro* was also a function of gender (figure 2). Cell characterization demonstrated that cells isolated from male samples have a tendency to differentiate *in vitro* without induction and express higher ratio of sarcomeric α-actinin and myosin heavy chain positive cells

(Figure 2A, $p<0.05$). This self-induced differentiation *in vitro* had no preference regarding muscle fiber type, as both slow and fast twitch fibers could be detected in higher levels in male than female samples. Despites these distinct differences cells from all biopsies could be characterized as muscle cells, with desmin and Pax 7 expression levels similar in all groups (Figure 2A, 2B and 2C). Upon induction of differentiation, cells of male donors fused and formed arranged myotubes faster than females (differentiation rate from female 16.6±1.4% and male 21.4±1.9%, Figure 2A and 2D, $p<0.05$). These cells expressed muscle sarcomeric structure already after 7 days after induction of muscle differentiation (figure 2E). Conversely, no significant differentiation ratio and myosin heavy chain expression variation could be detected among the age groups.

Human MPC engrafts progress its contractile response to electrical stimulation and improve innervations with time

To evaluate the impact of donor gender and age on the success of muscle bio-engineering, we have injected cultured cells in the subcutaneous space of nude mice and retrieved the engineered muscle after 2 and 4 weeks. Although engrafts sizes were not significant different, cell differentiation, myofiber formation and tissue contractility improved as a function of time (figure 3). Myogenic commitment, demonstrated by MyoD protein expression, of injected precursor cells increased nearly 4-fold between the second and fourth week of implantation (day14 0.99±0.11 and day28 3.90±0.66, $p<0.001$). Sarcomeric markers like sarcomeric α-actinin (day14 1.98±0.16 and day28 2.79±0.22, $p=0.007$) and Myosin heavy chain (day14 1.84±0.19 and day28 2.88±0.19, $p<0.001$) expression levels also increased with myofibers

adapted into slow and fast twitch fibers (figure 3D, 3E and 3F). Nerve ingrowth was also boosted as a function of time (day 14 0.73±0.11 and day 28 1.07±0.13, $p<0.05$).

If new myofibers and nerves are present in the engineered muscle tissue, its function and contractility should also be boosted. To assess if muscle function would fluctuate after transplantation, we have performed electromyography. While on the second week newly formed muscle fibers were still unorganized and with 539.6±94mg of contractile strength, on the fourth week, muscle structures were well organized and displayed nearly double contractile function (993.8±154mg). Histological analyses with H&E and desmin stainings demonstrated better organized muscle architecture after 4 weeks of transplantation (figure 3I).

Impact of Age – Muscle contractibility and fiber typing are a function of Age

All samples were able to form functional muscle fibers *in vivo*. We found no significant difference on Pax7 expression between different age groups (Figure 5A). However, commitment to myogenesis and expression of MyoD decreased as a function of age (21-40y 2.84±0.39, 41-60y 1.71±0.18 and 61-80y 1.27±0.28, figure 4B, $p<0.001$). Desmin expression was also significantly higher on engineered muscles from younger donor cells (Figure 4C). The expression of sarcomeric markers like Myosin heavy chain indicated that cell differentiation rebuilding mature muscular structures *in vivo* decreases with donor age (Figure 4D, $p=0.038$). The data specify still that cells isolated from young donors tend to develop a higher number of slow twitch fibres (Figure 4E, $p=0.034$). Hence, samples isolated from younger patients developed into stronger muscle fibers (21-40y 1464±306.1mg, 41-60y 1000±275.3mg and 61-80y 448±104.6mg, $p<0.05$, figure 4F) with a higher ratio of slow twitch myotubes (figure 4G).

Impact of Gender – Female cells maintain an undifferentiated state *in vitro*, are better innervated, and generate higher forces with preferentially slow twitch muscle fibers after transplantation

After transplanted a fraction of the implanted cells tended to build within the newly formed tissue a subpopulation of resident precursor cells. Although these subpopulations were similar in both gender one month after transplantation, male cells clearly tended to increase stem cell like (Pax7) characteristics and decrease sarcomeric protein expression (MyHC) after 4 weeks of transplant (figure 5A). Conversely, myogenic commitment (MyoD) remained higher in female cells suggesting a higher activity of precursor cells during muscle tissue formation. The ratio of Pax7 to MyoD has been described to reflect the cell commitment to myogenesis [22,23]. Cells from male donors displayed a 4-fold higher Pax7 to MyoD ratio at time of injection (female 0.38±0.28 and male 1.49±0.64). This ratio converted after transplantation and female samples display at day 14 8-fold higher (female 27.37±16.23 and male 3.24±1.15) and at day 28 4-fold higher (female 15.65±2.36 and male 3.54±0.53) myogenic activation ratio than male cells. These results suggest that cells from male donor commit early *in vitro* to myogenesis, decreasing their differentiation capability *in vivo*, whereas female cells are triggered to myogenesis mostly after transplantation.

The newly engineered muscle tissue also changed their characteristics with time after transplantation. Male cells decreased their sarcomeric proteins expression after transplantation and presented no advantage on building a new nerve network (figure 5A, 5B). Engineered muscle tissue from female donor cells displayed an increase in acetylcholine receptor clustering and in nerve filaments at days 14 and 28 after

transplantation (Figure 5B). Relative expression of PGP 9.5 was also superior on female donor samples at day 28 after transplantation (female 0.92±0.16 and male 0.58±0.11) (Figure 5C). In fact, the implanted cells from female patients were better innervated after 1 month of the transplant, directly reflecting in their response to electrical stimulation (figure 5D). Female cells engineered samples produced muscle tissues with increasing contraction force (821.6±107.4mg at day 14 and 1532.0±228.1mg at day 28, $p<0.001$), whereas engineered tissues derived from male cells decreased contraction strength (793.4.6±148.1mg at day 14 and 282.9±48.0mg at day 28, $p<0.001$) upon electrical tetanic stimulation (80V 80Hz).

Myogenic commitment and differentiation state at time of transplantation are decisive to the final contraction force of engineered muscle tissue

Despites the differences between genders all samples could generate functional and contractile tissue after transplant. However, the state of cell differentiation at transplantation time inversely correlated with the contractility of the engineered muscle tissue *in vivo*. Cells that at time of transplantation had a higher differentiation ratio developed into weaker muscle tissue (figure 6A). This inverse correlation could be observed in the different age groups (Pearson -0.834, $p<0.001$) as well as with samples of male and female provenience (Pearson -0.754, $p<0.001$).

Final engraft contractile response also inversely correlate to pre-transplant *in vitro* MyHC expression (Figure 6B, Pearson -0.781, $p<0.001$). This decrease in MyHC expression occurred mainly by fiber type switching into slow twitch fibers – MyH1 positive. Cells of female provenience had at time of transplantation lower MyH expression and were *in vivo* triggered to differentiate and form slow twitch muscle fibers (D0 0.35±0.14, D14 0.45±0.07, D28 2.67±0.47, Figure 6C, $p<0.001$).

Conversely, cells from male donors had a high MyH1 expression at time of transplantation, but decrease MyH1 upon time (D0 1.54±0.23, D14 1.46±0.26, D28 0.77±0.19, Figure 6C, $p<0.05$).

Discussion

Developing a novel autologous cell therapy is a highly complex matter that includes optimization of cell handling from biopsy to injection, legal application process for clinical trials, good medical practice (GMP) processing and patient education. Therefore, exploring the limitations and selecting the optimal patient population beforehand is critical to therapy long term success. Although the molecular mechanisms associated with cellular aging after isolation and culture growth leading to decline of cellular function as oxidative stress, mitochondrial dysfunction[24], DNA damage[25] and telomeres shortening[26] have been extensively described, the impact of the donor traits as age and gender for the success of human autologous muscle precursor cells transplants has not yet been clarified. In this study we isolated muscle precursor cells from human muscle biopsies of individuals between 21 and 80 year of age, of both sex, and investigated their cellular features and muscle regeneration capacity in culture and in a mouse model. This is the first study analyzing human donor trails as age and sex on the success of autologous muscle transplantation. We were able to confirm the myogenic phenotype, a great expansion potential and muscle fiber formation in all ages and both gender. However, remarkable differences between age and gender groups could be detected. These findings demonstrate that human MPCs are therapeutically useful for the treatment of muscle deficiencies but that the donor dissimilarities should be carefully considered.

It was long thought that gender and age merely play a role on muscle mass and its distribution on the body[16,27]. However, closer analyses into the muscular tissue revealed that resident precursor cells are essential for the different responses of muscle to injury as well as to resistance training associated muscle hypertrophy[9]. We demonstrated that the muscle biopsy size necessary to achieve the same MPC cell

number was double in young and females compared to elderly and males. Similarly, cells from young and females donors demonstrated an increased growth rate when compared to correspondent age and gender cell groups. Previous studies have described that the number of tissue resident MPCs are also considerable larger in young individuals[28] as well as in women, which correlates to a higher myofiber nuclei number upon injury[29]. Further, we reported that elderly and male derived cells grown under standardized conditions differentiate quicker *in vitro*. A recent study described that female Mesenchymal stem cells (MSCs) transplants were also more successful than male MSCs because male cells produce significantly greater tumor necrosis factor alpha, and less vascular endothelial growth factor than female cells[30]. We have previously reported that MPCs are also capable to produce TNFα, which blocks cell growth by inducing MPCs differentiation[31]. Taken together, these studies support our findings that cells from female and young donors may be a more efficient source of muscle cells and therefor the most promising for autologous muscle cell therapy.

Another decisive drawback of adult stem cells is their limited proliferative capacity and differentiation after long-term culture. Previous muscle gene expression studies have suggested significant differences only in muscle mass of different age and gender groups due to the influence of sexual hormones[16,27]. A study with muscle derived stem cells (MDSC) described that gender differences in muscle reconstruction capacity were not directly hormonal, but rather due to oxidative stress inducing *in vitro* differentiation of male cells[32]. We report that the reduced ability to grow functional muscle of male and elderly cells *in vivo* was at least partially explained by their early *in vitro* differentiation. The time needed to achieve enough cell number for transplantation was significantly longer in samples of male and elderly and although MPCs of all ages and both gender were able to form muscle *in vivo*, a gender- and age-dependent decline

in contractile response to electrical stimulation could be detected. Female cells were not only stable *in vitro*, but grown faster and produced better contraction upon electrical stimulation. Taken together, our results suggest that injecting cells in an undifferentiated state will probably yield the best functional muscle by allowing for further *in vivo* cell expansion followed by myofiber formation.

Fiber typing also reverted an important difference between age and gender corresponding groups in our study. While female cells differentiated into slow twitch fibers *in vivo*, male cells tended to decrease MyH1 expression. Further, muscle fiber typing was also age-related, with a decreasing MyH1 expression with increasing age. These findings coincided with the electrical stimulation response of the engineered tissue, where a weaker response to tetanic electrical stimulation correlated to a decreasing Myosin heavy chain type 1 expression. These findings are in line with a study on muscle adaptive response to exercise, which demonstrated that young females adapt muscle fibers to type 1 decreasing the amount of fibers type IIx, whereas male and older females increase fiber type II in response to resistance exercise[33]. In animal studies, it has been described that muscle maturation seems to be associated with increase in fiber size and nuclei number, whereas its atrophy seems to be correlated with decrease on muscle fiber types II[34]. Additionally, the age related satellite cell content reduction was specifically described in type II muscle fibers[35]. Taken together, these finding suggest that female and young MPC have an advantage in the formation of muscle tissue *in vivo* by increasing their proportion of muscle type 1 fiber, which support the survival of the satellite cell population.

We were able to demonstrate that human MPCs can be successfully isolated from a skeletal muscle biopsy and grown in standardized culture conditions achieving enough cell number for transplantation. Likewise, we could confirm the myogenic phenotype,

great expansion potential, fiber formation and the generation of a contractile muscle tissue after MPCs transplantation isolated from patients of all ages and both gender. In this study we identified intrinsic and important age and gender-related differences in MPCs from the initial isolation of MPCs until the final functional response of implanted engrafts to electrical stimulation. This unique features need to be considered when planning clinical trials for the treatment of muscle disabilities with MPCs.

References

1. Rando TA. Stem cells, ageing and the quest for immortality. *Nature*, 441(7097), 1080-1086 (2006).
2. Ho AD, Wagner W, Mahlknecht U. Stem cells and ageing. *EMBO Rep*, 6(S1), S35-S38 (2005).
3. Sabin RJ, Anderson RM. Cellular Senescence - its role in cancer and the response to ionizing radiation. *Genome integrity*, 2:7 (2011).
4. Hayflick L. How and why we age. *Experimental Gerontology*, 33(7–8), 639-653 (1998).
5. Hill M, Wernig A, Goldspink G. Muscle satellite (stem) cell activation during local tissue injury and repair. *Journal of Anatomy*, 203(1), 89-99 (2003).
6. Wozniak AC, Anderson JE. Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. *Developmental Dynamics*, 236(1), 240-250 (2007).
7. Zhan M, Jin B, Chen S-E, Reecy JM, Li Y-P. TACE release of TNFalpha mediates mechanotransduction-induced activation of p38 MAPK and myogenesis. *J Cell Sci*, 120(4), 692-701 (2007).
8. Hawke T, Garry D. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*, 91, 534 - 551 (2001).
9. Petrella JK, Kim J-s, Mayhew DL, Cross JM, Bamman MM. Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *Journal of Applied Physiology*, 104(6), 1736-1742 (2008).
10. Eberli D, Soker S, Atala A, Yoo JJ. Optimization of human skeletal muscle precursor cell culture and myofiber formation in vitro. *Methods*, 47(2), 98-103 (2009).
11. Asumda FZ, Chase PB. Age-related changes in rat bone-marrow mesenchymal stem cell plasticity. *BMC Cell Biology*, 12(44)).
12. Gibson MC, Schultz E. Age-related differences in absolute numbers of skeletal muscle satellite cells. *Muscle & Nerve*, 6(8), 574-580 (1983).
13. Strasser H, Tiefenthaler M, Steinlechner M, Eder I, Bartsch G, Konwalinka G. AGE DEPENDENT APOPTOSIS AND LOSS OF RHABDOSPHINCTER CELLS. *The Journal of Urology*, 164(5), 1781-1785 (2000).
14. Schultz E, Lipton BH. Skeletal muscle satellite cells: Changes in proliferation potential as a function of age. *Mechanisms of Ageing and development*, 20(4), 377-383 (1982).
15. Carlson ME, Conboy IM. Loss of stem cell regenerative capacity within aged niches. *Aging Cell*, 6(3), 371-382 (2007).
16. Gallagher D, Visser M, De Meersman RE *et al.* Appendicular skeletal muscle mass: effects of age, gender, and ethnicity. *Journal of Applied Physiology*, 83(1), 229-239 (1997).
17. Karakelides H, Irving BA, Short KR, O'Brien P, Nair KS. Age, Obesity, and Sex Effects on Insulin Sensitivity and Skeletal Muscle Mitochondrial Function. *Diabetes*, 59(1), 89-97 (2010).
18. Ng A, Callister R, Johnson D, Seals D. Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. *Hypertension*, 21(4), 498-503 (1993).
19. Pansarasa O, Castagna L, Colombi B, Vecchiet J, Felzani G, Marzatico F. Age and sex differences in human skeletal muscle: Role of reactive oxygen species. *Free Radical Research*, 33(3), 287-293 (2000).
20. Delo DM, Eberli D, Williams JK, Andersson K-E, Atala A, Soker S. Angiogenic gene modification of skeletal muscle cells to compensate for ageing-induced decline in bioengineered functional muscle tissue. *BJU International*, 102(7), 878-884 (2008).
21. Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and Eosin Staining of Tissue and Cell Sections. *Cold Spring Harbor Protocols*, 2008(5), pdb.prot4986 (2008).
22. Olguín HC, Patzlaff NE, Olwin BB. Pax7-FKHR transcriptional activity is enhanced by transcriptionally repressed MyoD. *Journal of Cellular Biochemistry*, 112(5), 1410-1417 (2011).
23. Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Developmental Biology*, 275(2), 375-388 (2004).
24. Hang Cui YK, and Hong Zhang. Oxidative Stress, Mitochondrial Dysfunction, and Aging. *Journal of Signal Transduction*, 2012 (2012).
25. Kenyon J, Gerson SL. The role of DNA damage repair in aging of adult stem cells. *Nucleic Acids Research*, 35(22), 7557-7565 (2007).
26. Hayflick L. A Brief History of the Mortality and Immortality of Cultured Cells. *The Keio Journal of Medicine*, 47(3), 174-182 (1998).

27. Gallagher D, Heymsfield SB. Muscle distribution: Variations with body weight, gender, and age. *Applied Radiation and Isotopes*, 49(5), 733-734 (1998).
28. Lees SJ, Rathbone CR, Booth FW. Age-associated decrease in muscle precursor cell differentiation. *Am J Physiol Cell Physiol*, 290(2), C609-615 (2006).
29. Kadi F, Thornell L-E. Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. *Histochemistry and Cell Biology*, 113(2), 99-103 (2000).
30. Crisostomo PR, Markel TA, Wang M, Lahm T, Lillemoe KD, Meldrum DR. In the adult mesenchymal stem cell population, source gender is a biologically relevant aspect of protective power. *Surgery*, 142(2), 215-221 (2007).
31. Stölting MNL, Ferrari S, Becskei A *et al.* Muscle Precursor Cells inhibit tumor growth upon secretion of TNF alpha. *in print*, (2012).
32. Deasy BM, Lu A, Tebbets JC *et al.* A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *J. Cell Biol.*, 177(1), 73-86 (2007).
33. Martel GF, Roth SM, Ivey FM *et al.* Age and sex affect human muscle fibre adaptations to heavy-resistance strength training. *Experimental Physiology*, 91(2), 457-464 (2006).
34. Bruusgaard JC, Liestøl K, Gundersen K. Distribution of myonuclei and microtubules in live muscle fibers of young, middle-aged, and old mice. *Journal of Applied Physiology*, 100(6), 2024-2030 (2006).
35. Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HHCM, van Loon LJC. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *American Journal of Physiology - Endocrinology And Metabolism*, 292(1), E151-E157 (2007).

FIGURES:

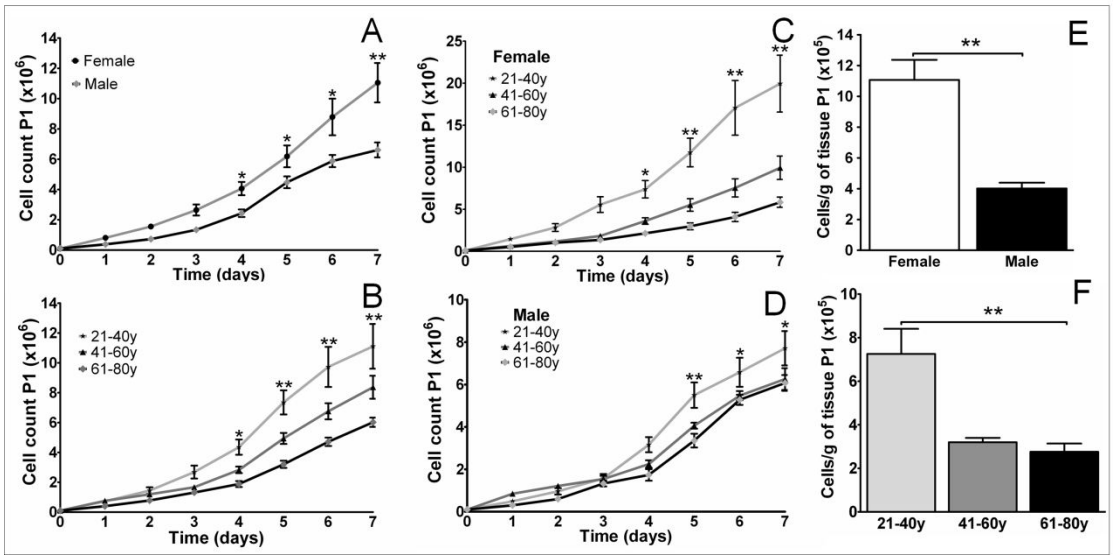

Figure 1 – **Influence of gender and age on MPC growth curves and cell yield per g of tissue**. MPCs were plated on a 24 well dishes at a density of 5000/cm^2 and daily cell counts (using a hemocytometer) were performed for all samples in passage 1. Although a standardized biopsy size was retrieved from individuals undergoing abdominal surgery, a different growth rate and cell recover per biopsy mass were detected. (A) Cells isolated from females and (B) young donors grown faster. Gender-dependent analyses demonstrated that age has an earlier impact on female than male cells. (C) A boosted growth could be observed in cells from female donors until 40 years of age, abruptly decreasing its importance between groups of 41-60 and 61-80 years of age. (D) Growth of male cells was also better in younger donor, but growth differences were frugal when compared to female samples. Similarly, cell yield per gram of muscle was higher in female and young donors. (E) Female muscle biopsies provided nearly the double of cells from a same biopsy size. (F) Cell yield decreased to a half after 40 years of age. *$p<0.05$, **$p<0.001$

A

	Pax 7	MyoD	Desmin	α-actinin	MyH	MyH1	MyH2	DR
Female	66.7±6.4	12.3±3.5	56.2±7.7	41.5±6.4	36.3±9.5	9.4±3.6	9.7±3.0	16.6±1.4
Male	65.4±7.0	48.4±5.2	69.8±5.8	68.5±6.4	70.7±6.1	85.6±3.7	56.4±9.6	21.4±1.9
p value	0.906	**<0.001**	0.089	**0.01**	**0.004**	**0.001**	**0.002**	**0.036**
21-40y	57.7±7.1	30.2±6.9	60.0±7.9	52.4±9.2	57.0±10.8	31.7±11.9	32.5±11.3	19.7±3.0
41-60y	76.4±10.2	44.8±10.9	74.7±11.8	69.0±10.7	81.6±5.8	59.0±5.4	61.1±17.8	16.4±1.88
61-80y	71.5±8.4	36.4±7.8	65.7±6.9	60.3±7.8	49.3±8.8	50.47±20.1	31.9±16.4	21.3±4.0
p value	0.272	0.538	0.548	0.509	0.137	0.554	0.523	0.648

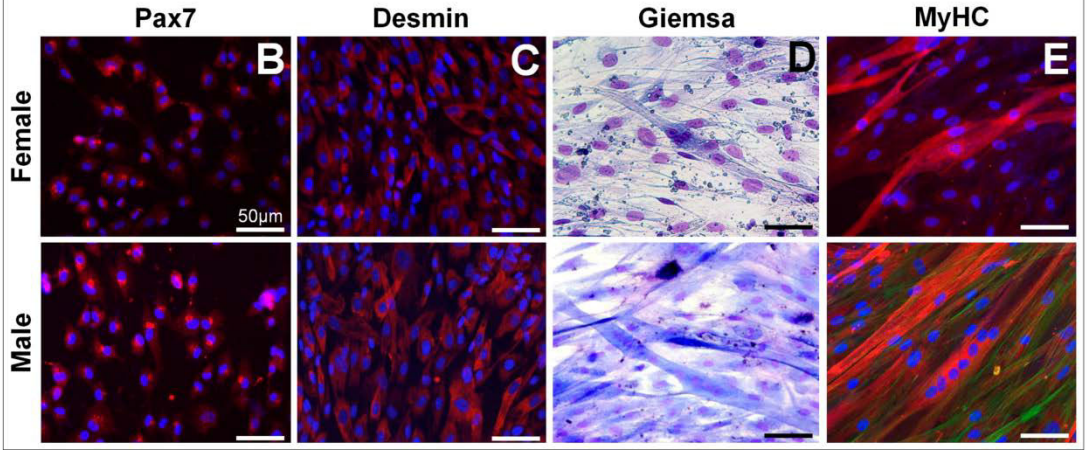

Figure 2 – **Influence of gender on the stem cell life spam and differentiation ratio**. Immunolabeled MPCs were analysed by FACS and immunocytochemistry to evaluate lineage specific markers. (A) Although no significant difference could be detected between age groups, a clear pattern could be verified between male and female cells. (A, B, C) The expression of Pax7 and Desmin was not different between gender groups. (A) However, a significant increase of MyoD and sarcomeric markers as sarcomeric α-actinin and MyHC could be detected in male cells ($p<0.001$, $p<0.05$, $p<0.05$ respectively). More male cells expressed both myosin types (G, H), whereas female cells remained undifferentiated at passage 3. (A, D, E) Immunocytochemistry confirmed the results of FACS analyses and Giemsa stained cells were measured by fiber formation assay determining a clear increase of differentiation ratio of male cells still *in vitro*. (I) DAPI (blue), mouse anti-PAX7, mouse anti-desmin and mouse anti-MyHC/anti-mouse IgG Cy3 (red). DR=Differentiation rate

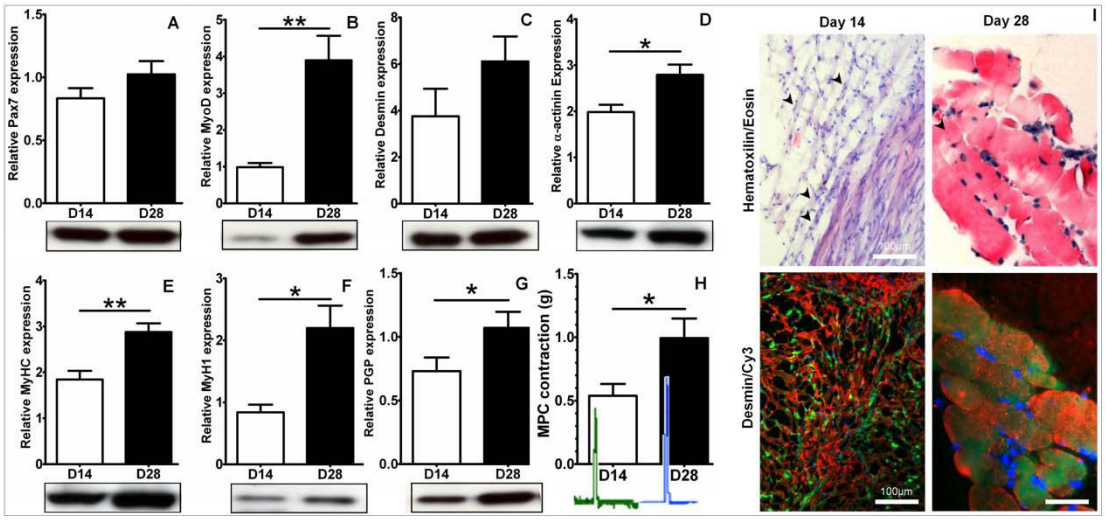

Figure 3 – **MPCs transplanted engrafts differentiate into muscle tissue and improve contractility with time.** All implanted MPCs samples were capable to establish engrafts *in vivo* with muscle fiber formation, innervation and contractile response to electrical stimulation. Time played an important role on morphology, protein expression and contractile function of engrafts. Our results demonstrate a time-dependent trend to increase the stem cell like population and Desmin expression (A, C), as well as a significant increase of myogenic differentiation (B). Protein semi-quantitative measurements confirmed that sarcomeric proteins as sarcomeric α-actinin (D), MyHC (E) and MyH1 (F) expression were also boosted as a function of time. (H) Muscle engrafts strength were measured by using dissected implants in a myograph and contractile response upon electrical stimulation doubled between the second and fourth week after injection. (I) Histological analyses with Hematoxilin/Eosin or immunostaining with Desmin demonstrated that muscle morphology differentiate into robust muscle fibers. The injected myoblasts were labelled *in vitro* with PKH 67 (green), and immunostaining was performed with DAPI (blue) and mouse anti-Desmin/anti-mouse IgG Cy3 (red). *p<0.05, **p<0.001

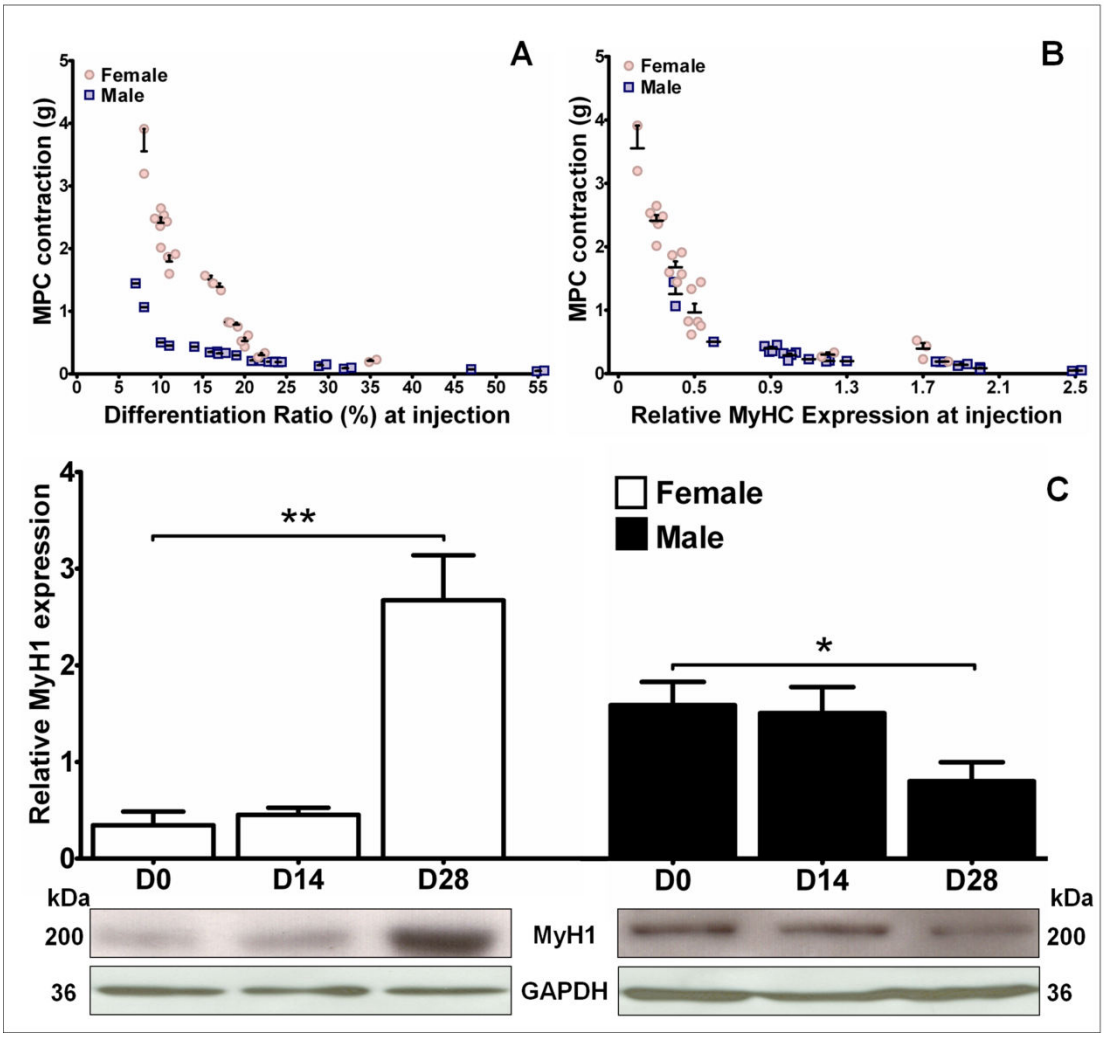

Figure 4 – **Age of donor has a deciding influence on the differentiation and contractile function of implanted MPCs**. Cells isolated from muscle biopsies of individuals grouped in three age groups were compared (21-40, 41-60 and 61-80 years of age). Morphology and protein expression were then analyzed as well as contractile function. (A) No significant difference could be found on the expression of the stem-cell like factor Pax7. (B) Commitment to myogenic lineage (MyoD) was increase in younger donors four days after transplantation. (C) Similarly, desmin expression was decreased in older donor cells. (D) Younger donors had cells that differentiate well *in vivo* with a higher expression of Myosin Heavy Chain (MyHC). (E) This seems to be a consequence of an increase in slow twitch fibers in youth donors (up-regulated MyH1 expression) and decrease in the elderly. (F) Consequently, elderly donors provided cells, which formed muscle tissue with lower contractile force. (G) To confirm Western blot results, the injected myoblasts were immunostained with DAPI (blue) and with mouse anti-MyH1 IgM/anti-mouse IgM FITC (green). Despites the contractile strength difference among groups, all engrafts were functional, producing contractile response after electrical stimulation. *$p<0.05$, **$p<0.001$

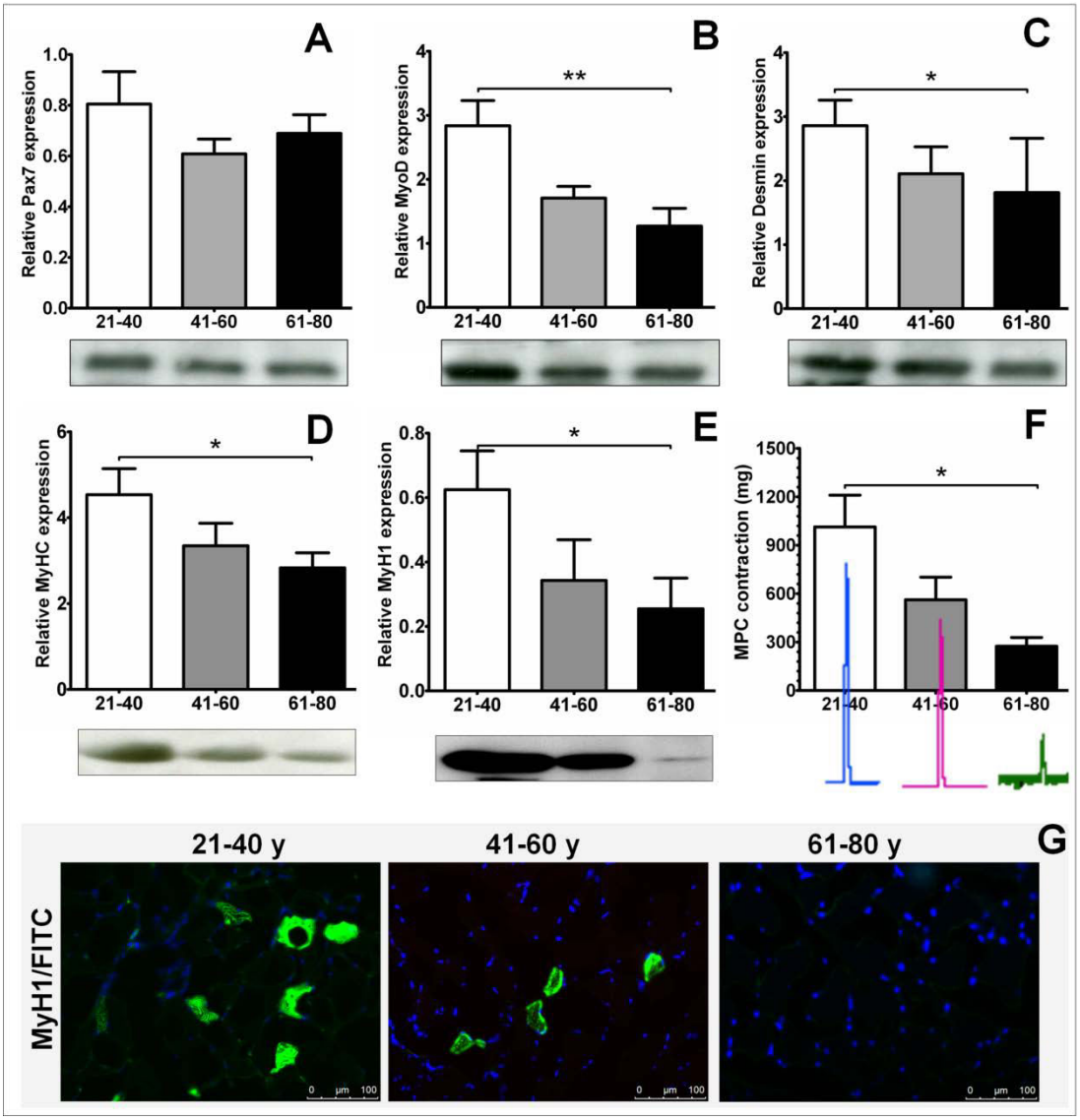

Figure 5 – **Donor gender is decisive in MPCs fate after transplantation –** Samples of both genders were analyzed and quantified by Western Blot and immunostaining on weeks two and four after transplantation. (A) Male cells become display increasing expression of early markers of myogenic differentiation and decreasing sarcomeric proteins with time. (B) Immunoshistochemistry of samples demonstrated that neuromuscular junctions (NMJ) and innervation increased over time in tissus of both donors. Samples were stained with DAPI (blue), mouse anti-NF68/Fluorescein (Green) and α-bungarotoxin (red). Red Arrow-heads point to acetylcholine receptor clusters. (C) Protein expression semi-quantitative measurements confirmed that female also prevail male samples regarding nerve ingrowth after 4 weeks of transplantation. (D) This differences in differentiation and nerve ingrowth had a direct impact on the myographic measured contractile capacity of engrafts, which decreased in tissue engineered from male and increased in samples from female donors (tetanic electrical stimulation 80V 80Hz). Dashed lines represent unpaired *t*-test significance levels comparing time points for the same gender. Continuous lines compare genders on the same time point. *p<0.05, **p<0.001

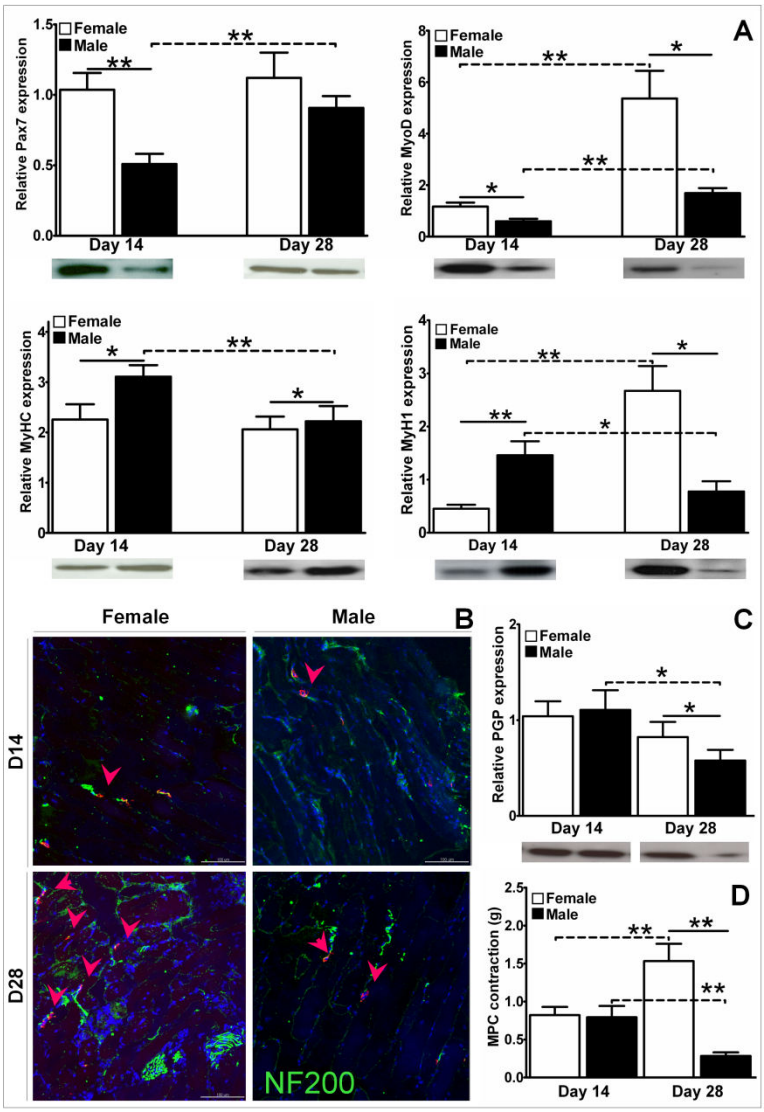

Figure 6 – **Differentiation ratio and MyHC expression at time of transplantation directly influence the final muscle contractile strength.** Although muscle cells received the same treatment in culture, donor traits played an important role on the spontaneous differentiation *in vitro*. The expression of sarcomeric proteins *in vitro* is crucial to define the quality of contractile response on newly formed MPC engrafts. We have evaluated engraft contractility strength with a myograph, differentiation rate by fiber formation assay and protein expression by Western Blot analysis. Cells from male and elderly donors demonstrated higher levels of differentiation in culture. At the same time, these sample groups led to lower contraction force after 4 weeks of cell transplantation. (A) Correlation plot shows that the intensity of muscle contraction (mg) upon electrical stimulation inversely correlated to the ratio of differentiation at time of transplantation. (Pearson= - 0.93, $p<0.001$). (B) Contractile response inversely correlate to MyHC expression of cells *in vitro* at time of transplantation (Pearson - 0.781, $p<0.05$). (C) MyH1 expression at time of cell injection and after 2 and 4 weeks of transplantation demonstrate that cells with female provenience differentiate in slow twitch fibers (MyH1 positive) with time, whereas male cells developed after 4 weeks *in vivo* into tissues with half of MyH1 expression encountered *in vitro*.

Title: **Magnetic Stimulation supports post-traumatic muscle tissue regeneration, induces nerve ingrowth and modulates neuromuscular junction formation and maturation**

Name of authors: **Meline N. L. Stölting[1], MD; Anne Sophie Arnold[2], PhD; Deana Haralampieva, M.Sc.; Christoph Handschin[2], PhD; Tullio Sulser[1], Prof.; Daniel Eberli[1]*, MD. PhD**

Name of institutions: [1]Laboratory for Urologic Tissue Engineering and Stem Cell Therapy, Division of Urology, University of Zurich, Frauenklinikstrasse 10, CH 8091 Zurich, Switzerland; [2]Biozentrum, Focal Area Growth and Development, University of Basel, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland.

* Corresponding Author: Daniel.Eberli@usz.ch, Phone: +41 44 255 9619, Fax: +41 44 255 9620

To Submit to: Neurorehabilitation and Neural Repair

Keywords:

Word count (Abstract): 207

Word count (text): 4320

Abstract

Magnetic stimulation (MS) has the ability to induce perceptible muscle twitch and has been long proposed as therapeutic modality for muscle skeletal diseases. Overtime different devices have been adapted, and all demonstrate its efficiency. However, the molecular mechanisms on how a magnetic field could induce contraction and its way of action have not been elucidated. We investigated the direct effect of MS on muscle and nerve regeneration *in vitro* and *in vivo*. MS supported early neuromuscular junction (NMJ) development. It induced muscle differentiation and nerve ingrowth, triggered synapses formation *in vitro*, which promoted the launch of isometric contractions ($p<0.001$). MS was found to support post-traumatic rehabilitation by significantly reducing the inflammatory infiltrate and scar size at the site of injury. It promoted hypertrophy of unscathed tissue, while avoiding post-trauma muscle atrophy. MS clearly increased the metabolism and turnover of muscle ($p<0.001$), triplicating the expression of desmin and myosin and inducing a shift to slow twitch fibers. Finally, MS is able to significantly promote acetylcholine receptor clustering and nerve ingrowth after injury, by inducing Agrin expression and thereby promoting NMJ maturation. Our results indicate that MS supports muscle and nerve regeneration by activating of the neuronal cellular machinery, increasing nerve-muscle cross-talk, inducing synapses and the maturation of NMJ.

Introduction

Historically developed to stimulate central and peripheral nerves[1, 2] magnetic stimulation (MS) has been proposed to promote neuromodulation and exercise training of deficient skeletal muscle[3, 4]. For instance for training of the lower limb a magnetic coil was wrapped around the quadriceps and connected to a transducer for measurements [4]. A similar device for exercising the pelvic floor was designed converging magnetic pulses into a coil placed inside of a chair seat[5]. The first one has been demonstrated to induce effortless muscle fatigue and training in human quadriceps[6]. The chair with a electromagnetic coil has been approved by the FDA/USA for the treatment of urinary incontinence in women[7, 8]. Although, promising preliminary clinical reports were published[9, 10], contradictory clinical studies with opposing outcomes on central[11, 12] and peripheral MS[13] treatment urged for an investigation demonstrating if and how MS could support the cellular machinery of muscle and/or nerves to promote muscle training[14].

MS has been investigated for rehabilitation and muscle conditioning exercise after spinal cord injury and muscle trauma. It is capable of inducing quadriceps maximal voluntary contraction[15], displays an age-independent effect[16], and is clinically applicable even after spinal cord injury[17]. Additionally, local MS is a superior alternative to electrical stimulation[18], which produces changes in muscle size, stimulated strength, eliciting equivalent muscle twitch amplitude without pain [19]. Attempts to explain these clinical findings suggested modulation of nerve synapses[20] with the necessity of a functional neuromuscular junction to induce the desired muscular training[21]. The influence of MS on axonal growth and the impact of MS on the NMJ development and muscle regeneration after trauma have not yet been established.

Nerve repair and prevention of post-traumatic muscle atrophy represent a major challenge in medical care. After trauma an initial cleanup of damaged structures is necessary before reconstruction can take place. Schwann cells are the main responsible for removing

damaged myelin, while macrophage infiltration is necessary for the crackdown of damaged fragments of cells and fibers[22]. Meanwhile, in the midst of the inflammatory reaction muscle satellite cells are activated for muscle reconstruction, they fuse and build new myofibers with central nuclei, allowing muscle regeneration within weeks[23]. The newly formed myofibers require rapid functional innervation and consequently mature neuromuscular junctions (NMJ) to complete their differentiation process and recover strength[24]. The presence of condition training is known to improve post-traumatic peripheral nerve lesion, improving function. MS is capable of producing endurance exercise in humans[4], but its mechanisms as well as its impact on post-traumatic muscle reconstruction remains uninvestigated.

In this study a neuromuscular junction co-culture model and a muscle crush injury mouse model are used to investigate the mode of action of MS on muscle tissue stimulation and post-trauma regeneration. The presence of synapses are confirmed during stimulation by evaluating the presence of neuronal depolarization, clustering of acetylcholine receptors (AChRs) and muscular metabolic adaptations caused by muscle contraction. MS influence on post-trauma muscle/nerve regeneration and vascularization is investigated in detail and its overall systemic impact and muscle fatigue are discussed.

Materials and Methods

Muscle cell isolation and culture

Human muscle samples were randomly collected upon ethical approval and with informed consent from the *rectus abdominis* of 10 hospitalized patients undergoing abdominal surgery under general anesthesia. Myoblasts isolation and culture were as previously reported[25]. After removing residual conjunctive tissue muscle biopsies were minced and digested in 0.4%/0.2% collagenase/dispase solution [1h, 37ºC], filtered (100µm strainer) and plated in 6well collagen-coated-dishes in a growth Medium (GM) containing DMEM/F12 medium(Gibco, Grand Island, NY), with 1% penicillin/streptomycin (Gibco), 18% FBS (Gibco), 10ng/ml hEGF (Sigma), 1ng/ml hbFGF (Sigma), 10µg/ml Insulin (Sigma) and 0.4 µg/ml dexamethasone (Sigma). A fibroblasts-reduction-step was performed by allowing the fibers digest to settle followed by replating to a new collagen-coated dish after 24h. Muscle cells were expanded until passage 2 (P2) with medium change every third day.

Chimerical Nerve-muscle co-culture model and magnetic stimulation in vitro

Human primary muscle cells were trypsinized and plated on 35-mm dishes (500,000 cells/dish) in GM. On the following day the medium was changed to differentiation Medium containing DMEM/F12 medium (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco), 10% fetal bovine serum (Gibco). After 2 days the nerve component was isolated from 13-day-old Wistar-rat-embryos and placed on top of the muscle cell layer. In detail, expecting females in day 13 of pregnancy were sacrificed (CO_2-asphyxiation), whole embryos were collected, decapitated and dissected under the microscope. A longitudinal incision was bilaterally made and the skin was removed. Spinal cords were isolated with the dorsal-root-ganglia still attached and transversally cut with 1 or 2 attached dorsal-root-ganglia. Each explant was gently placed on the surface of the muscle cell layer. Co-cultures were exposed to

3 MS sessions every second day (20 min, 50Hz, 3s stimulation, 6s rest) on the Biocon-2000W™. Non-stimulated co-cultures were used as controls. Medium was changed every third day.

Fiber Formation Assay (FFA) and neuritis length

Muscle differentiation ratio *in vitro* was calculated as previously reported[26]. Briefly, cells were seeded on slide chambers and, after 8 days in differentiating medium myofibers were fixed (methanol, 7min), stained (1:20 Giemsa, 1h) and air dried. Images were taken with a Leica-Imager-M1 Microscope. Five high-power-fields (HPF) per sample were analyzed (n=20) and results were expressed as differentiation rate, which was calculated by the number of nuclei in differentiated myofibers/HPF divided by the total number of nuclei/HPF. Images of unstained muscle-nerve co-cultures were also analyzed, and growth of neuritis from spinal cord slices was monitored on days 1, 3 and 5 after MS stimulation (n=15, 20x HPF). The NIH software "IMAGEJ for microscopy" software was used for measurements and all data are expressed as mean/SD.

Muscle Injury surgical procedure and magnetic stimulation in vivo

Forty-eight C57-mice (3-month-old females) under anaesthesia (5% isofluran) and aseptic conditions (shaved skin, betadine asepsis) received an upper-leg lateral incision (from the lateral knee to the greater trochanter)[27]. A coronal-plane beneath the quadriceps was opened separating the muscle from the femur. The lower jaw of an artery-forceps[28] was gently inserted below the quadriceps. Crush injury was performed by closing the forceps to its first stage for 5 seconds. Forceps was gently removed and the wound was closed. The animals were allowed free-cage-activity, free-access to food and water. Animals received Carprofen (5mg/kg/KGW, SC) before muscle injury and every 12h in any sign of pain. After 5 days animals were randomly divided in 2 groups. Twenty mice received 3 sessions of MS under

anaesthesia (2,00 mg/30 g Ketamin and 0,06 mg/30 g Azepromazine) every second day (20 min, 50Hz, 3s stimulation, 6s rest) on the Biocon-2000W™. The other 20 mice served as control and received no MS. To observe the effect of MS in the absence of trauma the remaining 8 mice received no muscle injury and half of them were submitted to MS.

Histological and histomorphometrical measurements

Harvested tissues were retracted after 5 days of MS therapy to evaluate muscle regeneration, muscle atrophy, myofiber typing and NMJ formation was previously described [29]. Briefly, quadriceps muscle was removed, minced and incubated in 0.2% collagenase (60min, 37°C). Single muscle fibers were liberated by shearing using heat-polished Pasteur-pipettes, washed in PBS, fixed (4% paraformaldehyde, 10 min), incubated in 0.3M glycine (20min), stained with α-BTX (1:20, Invitrogen) and DAPI (1:100, Sigma) for 1 hour, washed twice in PBS, transferred to a glass-slide in DABCO (Sigma) and analyzed by fluorescent microscopy. At a 63× magnification, 20 fields were randomly chosen, and the number of NMJs was counted. The relative number of AChR clusters per muscle fiber (50 muscle fibers per group) was analyzed.

For histological analyses, quadriceps muscles were dissected, embedded within OCT, and 10µm frozen sections were prepared and air dried. Hematoxilin/eosin staining was done as previously described[26]. Tissues were fixed (100% methanol, -20ºC), permeabilized (0,5% Triton X-100, 7min) and blocked (1% BSA, 0,1% Triton X-100 in PBS, RT, 30min). The Antibody concentration for Immunolabelling was anti-Desmin 1:100 (BD Biosciences), anti-myosin-heavy-chain 1:2 (DSHB, Yowa), anti-myosin-heavy-chain-slow-twitch 1:5 (DSHB, Yowa), anti-myosin-heavy-fast-twitch 1:2 (DSHB, Yowa), alpha-bungarotoxin (1:20, Invitrogen), anti-neurofilament 68 (1:300, Sigma), anti-von-Willenbrand-factor (1:50, abcam), anti-smoothelin (1:100, Santa Cruz). The secondary antibodies were 488-anti-mouse-IGG (1:100, Brunschwig), FITC-anti-mouse-IgM (1:100, sigma), Cy3-anti-mouse-IgG

(1:1000, Sigma), Cy3-anti-rabbit-IgG (1:100, Sigma), FITC-anti-Sheep IgG (1:100, Abcam) and DAPI (Sigma). Images were acquired at exposures that were based on unstained controls taken with a Leica SP5 confocal microscope.

Muscle regeneration was evaluated by counting fibers with centrally located nuclei of 20 randomly chosen sections in blinded fashion and normalizing by the total number of muscle fibers per field[30]. Inflammatory infiltrate was determined by the migration reach of inflammatory cells from the border of the injury scar. The percentage of slow-twitch and fast-twitch fibers was determined after immunostaining of type I fibers (FITC stained) and type II fibers (Cy3 stained). Muscle atrophy was assessed by measuring muscle fiber cross-sectional areas[31]. Unscathed and unstimulated (native) quadriceps muscle was used as a control for histology. The entire quadriceps muscle cross section was analyzed (n=20, 20x HPF), with care taken to ensure comparable cross section locations within and on the border of the induced muscle injury. The NIH software "IMAGEJ for microscopy" software was used for measurements and all data are expressed as mean/SD.

Serum creatinine, creatine kinase, bilirubin, myoglobin and haptoglobin

To evaluate possible systemic effects of MS on the mice we collected mouse blood and serum was isolated by centrifugation using heparin-coated collection tubes (BD Biosciences). Serum creatinine, creatine kinase, bilirubin, myoglobin and haptoglobin activity were then determined with specific-assays-kits according to the manufacturer's protocol (Diagnostic Chemicals Limited).

Western Blot

Culture nerve muscle constructs and harvested samples of the crushed zone we analysed by Western Blot (WB) as previously described[26]. In summary, tissues were pulverized in liquid nitrogen with mortar/pestle washed with PBS/protease inhibitor-cocktail (Sigma) and lysed

Protein lysates were measured using Pierce®BCA Protein-Assay-Kit (Thermo Scientific), loaded on 12% Biorad gels (30µg), transferred onto PVDF membranes (Immobilion-P; Millipore, Bedford, MA), blocked 1h in 5% non-fat-dry-milk, and incubated with primary antibodies at 4°C overnight. The primary antibodies were Mouse anti-Desmin (1:500, BD Biosciences), Mouse anti-myosin-heavy-chain (1:50, DSHB, Yowa), Mouse anti-myosin-heavy-chain I (1:25, DSHB, Yowa), Mouse anti-myosin-heavy-chain II (1:25, DSHB, Yowa), Mouse PGP9.5-Neuronal Marker (1:2000, Abcam), anti-neurofilament 68 (1:1000, Sigma), Rabbit anti-Agrin (1:200, Santa Cruz) and monoclonal anti-GAPDH (1:2000, Sigma). Membranes were washed in TBS/0.1% Tween-20 (30 min) and incubated 1h with the appropriate HRP-conjugated secondary antibody (Amersham, Dübendorf, Switzerland) in TBS/0.1% Tween-20/5% non-fat-dry-milk. Filters were developed by an ECL-technique (ECL-Kit, Amersham, Freiburg, Germany). Protein values were normalized by using the ratio target-protein/reference-gene (GAPDH). The quantification of each gene was performed using the NIH software "Image J" (NIH, Bethesda, MD).

RT-qPCR

RNA extraction, cDNA preparation and RT-qPCR reactions were done using Taqman® gene-expression-assay-kits (Applied Biosystems) for TNFαdesmin, MyH1, MyH2 and acetylcholinesterase, according to manufacturer's protocols. Reverse transcription conditions were: 25ºC for 10min, 37ºC for 120min, 85ºC for 5min. The data was normalized with 18S expression, quantitatively analyzed by measuring the threshold cycles (C_T) in a Microsoft Excel program and graphically on amplification plots. Fold changes were calculated as described previously[32].

Myography

For myographic examination, crush injury was performed in the *tibialis anterior* muscle. After harvest muscle was kept under tension with constant oxygenation (95% O_2 and 5% CO_2) in Krebs solution at room temperature. Muscle strips were fastened with vicryl into the myograph-chambers (DMT, Denmark) and aloud to equilibrate 20min under 20mN tension. Tension was adjusted and Kreb's solution replaced every 5 min. Single 80V/80Hz twitch stimulations were used to determine the optimum length (L0) of each tissue, and maximum tension under titanic contractions was registered. All data was collected using a LabChart v7.0 (ADinstruments, Spechbach, Germany) and expressed as mean/SD.

Statistics

All presented data are expressed as averages with corresponding standard deviation. For statistical analysis SPSS v11 (SPSS Inc, Chicago, IL) was used and graphics were drawn with GraphPad Prism v5.04 (GraphPad Software, Inc.). All data were analyzed by independent samples t-tests or one way ANOVA with *Bonferroni* post hoc analysis. A $p<0.05$ is considered significant.

Results

Magnetic stimulation induces nerve ingrowth, muscle differentiation and synapsis in vitro

To determine the effects of magnetic stimulation on the development of muscle and nerve, we have employed an established chimeric co-culture model of human myoblasts and embryonic rat spinal cord slices. Nerve ingrowth was estimated by length measurements of neurites, grown out of the spinal cord posterior ganglia in co-culture (Figure 1A, 1B). Their length to increase already after 1 day of stimulation (99.8±11.1µm, $p=0.116$), nearly doubled after 3 days (152.5±15.9µm, $p=0.002$) and increased up to 3-fold after 5 days (325.9±25.4µm, $p<0.001$) of MS treatment when compared to unstimulated controls 76.8±10.4µm, 83.0±8.6µm and 113.0±14.1µm respectively. Likewise, MS boosted the differentiation rate of myoblasts into myotubes from 25.9±2.8% to 44.0±3.0% (Figure 1C, $p<0.001$). RT-qPCR confirmed this finding by displaying a 3-fold increase of TNFα RNA expression, indicating that myoblasts were induced to differentiate[26] (Figure 1D, $p<0.001$). Further, the up-regulation of muscle specific markers as Desmin (figure 1E, $p<0.001$) and Myosin Heavy Chain 1 and 2 (figure 1F, 1G, $p<0.001$) demonstrated the induced changes in muscle phenotype. Finally, the presence of functional synapses could be assured by the increase on acetylcholinesterase (AChE) expression (Figure 1H, $p=0.034$).

Magnetic stimulation improves muscle contractile function inducing myofiber hypertrophy

To investigate the role of magnetic stimulation on the contractile response of muscle we made use of nerve-muscle co-cultures and observed their residual spontaneous contractile profile immediately after MS treatment. We found that MS is sufficient to reduce contractile frequency from a fibrillating and unstable 3.6 contractions per second to an efficient and rhythmic 2 contractions per second after MS stimulation (figure 2A, 2B, 2C, Supplementary videos). The diameter of contracting muscle fiber was measured in relaxed state (control

15.24±3.93 µm and treated 26.33±3.40µm, p<0.001, figure 2D) and during contraction (control 20.77±3.05 µm and treated 38.15±4.56µm, p<0.001, figure 2E). The twitch capability (Δdiameter) quantified at each contraction was significantly higher on the MS stimulated samples (control 20.77±3.05 µm and treated 38.15±4.56µm, p<0.001, figure 2E). We found that the muscle fibers after stimulation display a training induced hypertrophy (control 22.6±1.6 µm and treated 37.1±2.2µm, p<0.001).

Magnetic stimulation causes no systemic or muscle damage but improves muscle regeneration by reducing inflammatory infiltrate

After 5 days of MS treatment muscle and blood samples were collected and compared to unstimulated controls (Figure 3A, 3B). To evaluate the systemic effects of MS *in vivo* circulating blood concentrations of creatinine, creatine kinase, bilirubin, myoglobin and haptoglobin were measured. The levels of haptoglobin, bilirubin, creatinine were below the reference limit of our assays indicating the absence of hemolysis, hepatic or kidney damage in both groups. Myoglobin circulating levels (Figure 3C) were similar and slightly elevated in both, stimulated and control animals (24.6±2.3µg/l and 25.2±1.3 µg/l respectively). Conversely, the levels of creatine kinase increased 5-fold in stimulated samples (control 193.5±90.36 U/l and 933.3±10.54 U/l respectively). This finding associated with normal myoglobin levels indicate the presence of exercise without additional muscle damage after MS (Figure 3D).

Histomorphometrical analysis of the injury site (Figure 3E, 3F, 3G) demonstrate that MS treated samples reduced post-traumatic scar formation (214.2±57.8µm) to a third of control values (686.3±71.8µm, p<0.001). MS also limited the inflammatory infiltrate to 347.4±18.9µm of the injury scar, while control samples displayed inflammatory cells even at 637.5±64.02µm of the scar interface (p<0.001). In addition, we assessed the presence of neovascularization by comparing double-staining of von Willenbrand factor (vWf) and

smoothelin (Figure 3I, 3J). As expected, the inflamed injury site had an increased vascularization (vWf 162±19.24%, p=0.003 and Smoothelin 190.8±24.42%, p=0.001). However in MS treated samples the number of newly formed vessels within the crush scar was back to similar values (vWf 96.33±6.35% and Smoothelin 97.93±25.04%) of unscathed control muscle.

Magnetic stimulation avoids post-trauma muscle atrophy and induces hypertrophy of unscathed tissue

Muscle fiber cross-section measurements were performed within the injury region (figure 4A, 4B) and at the interface to normal muscle (figure 4E, 4F). We found that myofibers within the injury region tended to decrease their cross-section to about 38.56±1.64% ($p<0.001$, figure 4C) of an intact quadriceps myofiber, whereas MS stimulated muscle did not undergo atrophy, remaining with fiber cross-section values similar or bigger than unharmed unstimulated controls (114.4±5.2%, $p<0.05$). Furthermore, the muscle regenerative process, demonstrated by the presence of myotubes with central nuclei disposition, was boosted ($p<0.001$) by the treatment with MS (figure 4D). A total of 80.67±6.96% of the muscle fibers in the injury site of MS-treated samples displayed central nuclei. Untreated muscle fibers exhibited only 41.54±8.11% of regenerating myofibers.

Additional analyses of tissue at the injury interface demonstrated that muscle adjacent to the trauma location became atrophic. Their muscle fiber cross-sections were reduced to 69.9±8.5% of the control (p=0.002, figure 4E, 4G). MS treatment was sufficient to prevent this process not only by inducing hypertrophy (134.5±6.5%, $p<0.001$) but also by boosting the regenerative process (Figure 4F, 4G). At the interface to normal muscle we found that 50.0±7.2% of the MS treated myofibers display central nuclei, while only 20.10±1.96% of the untreated samples were regenerating (Figure 4H).

Magnetic stimulation induces shift of muscle fiber type to slow-twitch and improves muscle contractile force

Analyses of immunostained muscle slices and semi-quantitative protein measurements were performed to estimate the impact of MS on muscle protein expression and fiber typing. Muscle specific proteins as Myosin Heavy Chain (MyH) tended to increase (control 0.43±0.06, stimulated 0.74±0.26, $p=0.056$) and Desmin (Figure 5A) doubled its expression after 5 days of magnetic stimulation (control 0.78±0.25, stimulated 1.6±0.26, $p=0.021$). Our Western blot (WB) results indicate a 3-fold increase in MyH1 (control 0.42±0.11 and stimulated 1.46±0.31, $p<0.001$), whereas no significant increase of MyH2 expression (control 0.11±0.10 and stimulated 0.19±0.07, $p=0.09$) after stimulation could be detected (Figure 5B, 5C). Immunostaining with MyH1/MyH2 confirmed the WB data, demonstrating that MS boosted MyH type 1 expression (figure 5D, 5E, 5F) when compared unstimulated control (128.3±24.4, $p<0.05$) and unscathed quadriceps (278.8±35.6, $p<0.001$). A consequent decrease in MyH type 2 (control 103.4±13.44, $p=0.2643$ and stimulated 68.56±9.31, $p<0.001$) after MS (figure 5D, 5E, 5F) also points out to fiber type shift to slow twitch.

Myography of native and damaged muscle tissue demonstrated that MS improves muscle contractile response to electrical stimulation. In the absence of trauma, tetanic contraction forces significantly increase after MS (control 3.34±0.67g and stimulated 5.73±1.18g, $p<0.05$, Figure 5H). Likewise, muscle strength recovery significantly improves after a crush injury if a MS treatment is applied (control 1.03±0.27g and stimulated 2.47±0.21g, $p<0.001$), suggesting that MS may be useful for muscle rehabilitation after trauma.

Magnetic stimulation promotes and nerve ingrowth after injury acetylcholine receptor clustering

To verify the influence of MS in recovering of innervation after injury we have analyzed the regenerating muscle tissue by evaluating the muscle components and NMJ formation. We

have found evidence of cross-talk between muscle and nerves (Figure 6A) promoting the maturation of neuromuscular junctions (NMJs). Levels of Agrin were significantly higher in MS-treated samples (control 0.70±0.17 and treated 1.06±0.13, p=0.046). Likewise, the expression of neurofilament protein NF68 (control 0.71±0.10 and treated 1.26±0.16, p=0.026) and PGP 9.5 (control 0.47±0.12 and treated 0.78±0.15, p=0.003) were up-regulated (Figure 6B, 6C). Moreover, the total number of acetylcholine receptors (AChR) clusters per high power field (HPF) was clearly higher in MS-treated samples (control 3.86±0.62 and treated 7.13±1.15, p=0.021, Figure 6D, 6E, 6F). Furthermore, the innervation coefficient of stimulated samples was not only higher than that of the MS-untreated samples (control 40.75±4.02 and treated 143.9±21.73, p<0.001) but it trespassed (p=0.036) even the coefficient found in a normal quadriceps muscle (Figure 6G, 6H, 6I). Finally, AChR displayed a better organization and distribution around the MS treated muscle fibers (control 0.07±0.04 and treated 0.61±0.09, p<0.001), increasing up to 8-fold the total number of clusters per muscle fiber (Figure 6J, 6K, 6L).

Discussion

Magnetic fields can be used to induce current forces that trigger muscle contraction. However, clinical studies remain controversial and no mechanism of action has been described. We have studied the effects of MS on muscle, neuromuscular junction and nerve *in vitro* by employing a nerve-muscle co-culture system. Further, we investigate *in vivo* the impact MS treatment on post-traumatic muscle and nerve regeneration, as well as, on the inflammatory infiltrate and neovascularization of the injury site. We have found that MS a) induces nerve ingrowth and muscle differentiation *in vitro*; b) causes no systemic or muscle damage but mimics the effects of exercise; c) improves muscle regeneration by reducing inflammatory infiltrate and avoiding post-trauma muscle atrophy; d) improves muscle contractile function by inducing myofiber hypertrophy; e) promotes acetylcholine receptor clustering and nerve ingrowth after injury; f) induces muscle fiber type switch to slow-twitch.

This is the first study that employing a nerve-muscle co-culture model under MS demonstrates the growth of neurites, increase of AChE and Agrin accumulation, leading to clustering of AChR and the consequent metabolic effects on muscle cells. Computational experiments have suggested that MS might induce somatic depolarization[33]. Others have demonstrated that after nerve sectioning no visible muscle contraction is present after MS indicating that it prompts muscle training by neuronal stimulation[34]. Additionally, clinical reports describe that MS promotes acute improvement on urinary incontinence symptoms after surgical sphincter damage improving [35]. Our results show that MS acts in the very beginning of early NMJ formation by promoting the cross-talk between muscle cells through Agrin liberation. Agrin is a heparin sulfate proteoglycan that activates muscle specific kinase (MuSK) to cluster cholinergic receptors on the post-synaptic plate[36]. It acts as an envoy between nerve and muscle initiating the cascade that promotes NMJ maturation and regulates synaptic function[37]. Our results propose that the faster neuronal ingrowth detected in MS treated tissues is also associated to the increase of Agrin. This finding went hand in hand with

muscle differentiation and the accumulation of AChE, which is known to be associated with the presence of a functional NMJ[38]. Finally, our results indicate that MS mimics the effects of endurance exercise, which increases turn-over and causes hypertrophy in skeletal muscle[39]. Taken together these findings indicate that MS induces neuronal ingrowth and myoblast differentiation in culture by promoting muscle-nerve cross-talk, inducing the maturation of NMJ and triggering synapses.

MS plays an important role on post-traumatic skeletal muscle regeneration. A previous report has suggested that MS might facilitate the regeneration process of skeletal muscle damage induced by mepivacaine[40]. However, this anesthetic is known to produce muscle fiber injury sparing vascular bed, nerve endings and satellite cells[41] and therefore not comparable to the damage caused after trauma. We have demonstrated the post-trauma impact of MS treatment efficiently promoting muscle regeneration, nerve ingrowth and AChR clustering in a mouse model after muscle crush injury. MS decreased the inflammatory infiltrate, avoided myofibers atrophy and boosted the muscle turn-over by increasing the number of regenerating fibers. If MS could have an impact in established nerve connection or even in chronically ill subjects remain to be studied MS did raise the cross-section of these fibers and even supported new fiber formation within healthy regions of the muscle. We have also observed that MS plays a decisive role in the trophy of unscathed muscle fibers adjacent to a site of injury, inducing phenotype changes compatible with the effects of exercise. Recent studies demonstrate that magnetic field is capable of inducing myoblast differentiation[42] and of promoting myotubes hypertrophy[43]. The increase of myofiber with central nuclei, demonstrated at the injury interface of MS stimulated samples, is important to support the regeneration process of the adjacent damaged tissue. This finding added to the hypertrophic state of MS treated muscles would explain the decrease in size of injury scar of the stimulated samples, by avoiding atrophy of the newly formed muscle fiber and inducing hypertrophy of unscathed tissue. We speculate that MS has the potential to induce differentiation of resident

satellite cells *in situ* as we observed *in vitro.* This is important because it indicates that MS acts not only in a site of injury, but also in the adjacent tissue working together to improve regeneration and muscle rehabilitation after trauma.

A major characteristic that makes MS an interesting rehabilitation treatment modality is its capability to excite a specific target in a painless and non-invasive manner. There is no study demonstrating whether MS would provoke damage of internal organs. In our system we placed the mouse directly on the MS coil and although the whole body of the animal was exposed to the alternating magnetic field we could find no sign of systemic damage. Only creatine kinase levels increased, which in absence of other positive rhabdomyolysis markers indicates the presence of muscle workout than damage[44]. After endurance exercise CK is expected to raise at least 3-fold higher than in resting condition[45]. Earlier studies support our findings by establishing that MS causes less damage than electrical stimulation to muscle, inducing a limited rise of creatine metabolites[21] . It has also been used in patients with chronic disease as Chronic Obstructive pulmonary disease[4], multiple esclerose[46], hypertension[47] and even during pregnancy[48] without complications. This is a vital piece of information that points out to the safety of MS treatment.

With the settings used MS was able to distinctly steer muscle fiber type decision. Although in early stages of muscle differentiation MS induced a rather general increase of both MyH type 1 and 2 (*in vitro* experiments), we found that during the regeneration process *in vivo* newly formed fiber would tend to shift their fiber type to slow twitch (MyH1). It is described that during the regenerative process newly formed myotubes tend to follow the intrinsic typing characteristics of the prior fibers[49]. Even though quadriceps is mainly a fast-twitch type of muscle[50], we detected a 3-fold increase on type 1 fibers after MS treatment. We have demonstrated that MS acts in the early development of NMJ by promoting myotubes formation and neuronal ingrowth, leading to AChR clustering and culminating in functional synapses capable to generate isometric muscle contractions. Additionally, our results propose

MS as an efficient support to post-trauma rehabilitation of acute nerve and muscle damage. Further systematic studies exploring the effects of MS in post-traumatic chronically damaged skeletal muscle would complete our understanding of the mechanisms by which MS acts on limb crush injury functional rehabilitation.

Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article

Acknowledments

We gratefully acknowledge the technical assistance of Dr. Sousan Salemi and Fatma Kivrak on the improvement of our FACS, IHC and WB methods. This work was supported by the Hartmann-Müller Foundation, Forschungskredit, Swiss National Foundation and University of Zürich.

Funding

The authors disclosed receipt of the following financial support for the research and/or authorship of this article:

Forschungskredit, Swiss National Foundation and University of Zürich.

References

[1] Barker AT, Jalinous R, Freeston IL. NON-INVASIVE MAGNETIC STIMULATION OF HUMAN MOTOR CORTEX. The Lancet. 1985;325(8437):1106-7.

[2] Bickford RG, Guidi M, Fortesque P, Swenson M. Magnetic stimulation of human peripheral nerve and brain: response enhancement by combined magnetoelectrical technique. Neurosurgery. 1987;20(1):110-6.

[3] POLKEY MI, LUO Y, GULERIA R, ÅRD C-HH, GREEN M, MOXHAM J. Functional Magnetic Stimulation of the Abdominal Muscles in Humans. American Journal of Respiratory and Critical Care Medicine. 1999 August 1, 1999;160(2):513-22.

[4] Swallow EB, Gosker HR, Ward KA, Moore AJ, Dayer MJ, Hopkinson NS, et al. A novel technique for nonvolitional assessment of quadriceps muscle endurance in humans. Journal of Applied Physiology. 2007 September 2007;103(3):739-46.

[5] D.D. Chandi PMG, P. L. Venema. Functional extracorporeal magnetic stimulation as a treatment for female urinary incontinence: 'the chair'. BJU International. 2004;93(4):539-42.

[6] Taylor JL. Magnetic muscle stimulation produces fatigue without effort. Journal of Applied Physiology. 2007 September 2007;103(3):733-4.

[7] Quek P. A critical review on magnetic stimulation: what is its role in the management of pelvic floor disorders? Current Opinion in Urology 2005;15(4):231-5.

[8] Petra J. Voorham2013Van Der Zalm ROBCMPAMSHWEGABLÀN. Effects of magnetic stimulation in the treatment of pelvic floor dysfunction. BJU International. 2006;97(5):1035-8.

[9] Hoşcan MB, Dilmen C, Perk H, Soyupek S, Armağan A, Tükel O, et al. Extracorporeal Magnetic Innervation for the Treatment of Stress Urinary Incontinence: Results of Two-Year Follow-Up. Urologia Internationalis. 2008;81(2):167-72.

[10] Choe J, Choo M-S, Lee K-S. Symptom change in women with overactive bladder after extracorporeal magnetic stimulation: a prospective trial. International Urogynecology Journal. 2007;18(8):875-80.

[11] Mitchell WK, Baker MR, Baker SN. Muscle responses to transcranial stimulation in man depend on background oscillatory activity. The Journal of Physiology. 2007 September 1, 2007;583(2):567-79.

[12] Ellaway PH, Davey NJ, Maskill DW, Rawlinson SR, Lewis HS, Anissimova NP. Variability in the amplitude of skeletal muscle responses to magnetic stimulation of the motor cortex in man. Electroencephalography and clinical Neurophysiology. 1998;109:104-13.

[13] Wallis MC, Davies EA, Thalib L, Griffiths S. Pelvic Static Magnetic Stimulation to Control Urinary Incontinence in Older Women: A Randomized Controlled Trial. Clinical Medicine & Research. 2012 February 1, 2012;10(1):7-14.

[14] Petra J. Voorham VDZ, R. O. B. C. M. Pelger, Anne M. Stiggelbout, Henk W. Elzevier, Guus A. B., Lycklama À Nijeholt. Effects of magnetic stimulation in the treatment of pelvic floor dysfunction. BJU International. 2006;97(5):1035-8.

[15] Kremenic IJ, Ben-Avi SS, Leonhardt D, McHugh MP. Transcutaneous magnetic stimulation of the quadriceps via the femoral nerve. Muscle & Nerve. 2004;30(3):379-81.

[16] Kirschner-Hermanns R, Jakse G. Magnetstimulation des Beckenbodens beim älteren Menschen. Der Urologe A. 2007;46(4):377-81.

[17] Khedr EM, Alkady EAM, El-Hammady DH, Khalifa FAM, bin-Humam S. Repetitive Lumbosacral Nerve Magnetic Stimulation Improves Bladder Dysfunction Due to Lumbosacral Nerve Injury. Neurorehabilitation and Neural Repair. 2011 July/August 2011;25(6):570-6.

[18] Szecsi J, Götz S, Pöllmann W, Straube A. Force–pain relationship in functional magnetic and electrical stimulation of subjects with paresis and preserved sensation. Clinical Neurophysiology. 2010;121(9):1589-97.

[19] Machetanz J, Bischoff C, Pichlmeier R, Riescher H, Meyer B-U, Sader A, et al. Magnetically induced muscle contraction is caused by motor nerve stimulation and not by direct muscle activation. Muscle & Nerve. 1994;17(10):1170-5.

[20] McFarlane JP, Foley SJ, Winter PD, Shah PJR, Craggs MD. Acute suppression of idiopathic detrusor instability with magnetic stimulation of the sacral nerve roots. British Journal of Urology. 1997;80(5):734-41.

[21] Atsushi Chiba MI. Phosphate metabolites in muscular contraction caused by magnetic stimulation. Bioelectromagnetics. 2003;24(5):366-71.

[22] Sarikcioglu L, Yaba A, Tanriover G, Demirtop A, Demir N, Ozkan O. Effect of Severe Crush Injury on Axonal Regeneration: A Functional and Ultrastructural Study. J reconstr Microsurg. 2007 04.05.2007;23(03):143-9.

[23] Chargé SBP, Rudnicki MA. Cellular and Molecular Regulation of Muscle Regeneration. Physiological Reviews. 2004 January 1, 2004;84(1):209-38.

[24] Lin S, Landmann L, Ruegg MA, Brenner HR. The Role of Nerve- versus Muscle-Derived Factors in Mammalian Neuromuscular Junction Formation. J Neurosci. 2008 March 26, 2008;28(13):3333-40.
[25] Stölting MNL, Ferrari S, Becskei A, Handschin C, Sulser T, Eberli D. Muscle Precursor Cells inhibit tumor growth upon secretion of TNF alpha. in print 2012.
[26] Stölting MNL, Ferrari S, Becskei A, Handschin C, Provenzano M, Sulser T, et al. Muscle Precursor Cells inhibit tumor growth upon secretion of TNF alpha. Journal of Urology 2012;in print.
[27] Jonathan R. Bunn JC, George Burke, Moses Mushipe, David R. Marsh, Gang Li,. Production of consistent crush lesions in murine quadriceps muscle - A biomechanical, histomorphological and immunohistochemical study. Journal of Orthopaedic Research. 2004;22(6):1336-44.
[28] Collins RA, Grounds MD. The Role of Tumor Necrosis Factor-alpha (TNF-{alpha}) in Skeletal Muscle Regeneration: Studies in TNF-{alpha}(-/-) and TNF-{alpha}(-/-)/LT-{alpha}(-/-) Mice. J Histochem Cytochem. 2001 August 1, 2001;49(8):989-1002.
[29] Handschin C, Kobayashi YM, Chin S, Seale P, Campbell KP, Spiegelman BM. PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. Genes & Development. 2007 April 1, 2007;21(7):770-83.
[30] Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. Foxo Transcription Factors Induce the Atrophy-Related Ubiquitin Ligase Atrogin-1 and Cause Skeletal Muscle Atrophy. Cell. 2004;117(3):399-412.
[31] Sandri ML, Jiandie; Handschin, Christoph; Yang, Wenli; Arany, Zoltan P.; Lecker, Stewart H.; Goldberg, Alfred L.; Spiegelman, Bruce M. PGC-1 alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proceedings of the National Academy of Sciences. 2006 October 31, 2006;103(44):16260-5.
[32] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.
[33] Pashut T, Wolfus S, Friedman A, Lavidor M, Bar-Gad I, Yeshurun Y, et al. Mechanisms of Magnetic Stimulation of Central Nervous System Neurons. PLoS Comput Biol. 2011;7(3):e1002022.
[34] Ellaway PH, Rawlinson SR, Lewis HS, Davey NJ, Maskill DW. Magnetic stimulation excites skeletal muscle via motor nerve axons in the cat. Muscle & Nerve. 1997;20(9):1108-14.
[35] Yokoyama TN, Jun; Watanabe, Toyohiko; Nose, Hiroyuki; Nozaki, Kunihiro; Fujita, Osamu; Inoue, Miyabi; Kumon, Hiromi. Comparative study of effects of extracorporeal magnetic innervation versus electrical stimulation for urinary incontinence after radical prostatectomy. Urology. 2004;63(2):264-7.
[36] Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee K-F. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature. 2001;410(6832):1057-64.
[37] Daniels MP. The role of agrin in synaptic development, plasticity and signaling in the central nervous system. Neurochemistry International. (0).
[38] Anglister L. Acetylcholinesterase from the motor nerve terminal accumulates on the synaptic basal lamina of the myofiber. The Journal of Cell Biology. 1991 November 1, 1991;115(3):755-64.
[39] Burd NA, Tang JE, Moore DR, Phillips SM. Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. Journal of Applied Physiology. 2009 May 2009;106(5):1692-701.
[40] Jimena I, Tasset I, Lopez-Martos R, Rubio AJ, Luque E, Montilla P, et al. Effects of Magnetic Stimulation on Oxidative Stress and Skeletal Muscle Regeneration Induced by Mepivacaine in Rat. Medicinal Chemistry 2009;5(1):44-9
[41] Okland S, Komorowski TE, Carlson BM. Ultrastructure of mepivacaine-induced damage and regeneration in rat extraocular muscle. Investigative Ophthalmology & Visual Science. 1989 July 1, 1989;30(7):1643-51.
[42] Stern-Straeter J, Bonaterra GA, Kassner SS, Faber A, Sauter A, Schulz JD, et al. Impact of static magnetic fields on human myoblast cell cultures International Journal of Molecular Medicine. 2011;28(6):907-17.
[43] Coletti D, Teodori L, Albertini MC, Rocchi M, Pristerà A, Fini M, et al. Static magnetic fields enhance skeletal muscle differentiation in vitro by improving myoblast alignment. Cytometry Part A. 2007;71A(10):846-56.
[44] ROSS JH, ATTWOOD EC, ATKIN GE, VILLAR RN. A Study on the Effects of Severe Repetitive Exercise on Serum Myoglobin, Creatine Kinase, Transaminases and Lactate Dehydrogenase. QJM. 1983 March 20, 1983;52(2):268-79.
[45] Totsuka M, Nakaji S, Suzuki K, Sugawara K, Sato K. Break point of serum creatine kinase release after endurance exercise. Journal of Applied Physiology. 2002 October 1, 2002;93(4):1280-6.
[46] de Carvalho MLL, Motta R, Konrad G, Battaglia MA, Brichetto G. A randomized placebo-controlled cross-over study using a low frequency magnetic field in the treatment of fatigue in multiple sclerosis. Multiple Sclerosis Journal. 2012 January 1, 2012;18(1):82-9.

[47] Cogiamanian F, Brunoni AR, Boggio PS, Fregni F, Ciocca M, Priori A. Non-invasive brain stimulation for the management of arterial hypertension. Medical Hypotheses. 2010;74(2):332-6.
[48] Zhang D, Hu Z. RTMS may be a good choice for pregnant women with depression. Archives of Women's Mental Health. 2009;12(3):189-90.
[49] Kalhovde JM, Jerkovic R, Sefland I, Cordonnier C, Calabria E, Schiaffino S, et al. 'Fast' and 'slow' muscle fibres in hindlimb muscles of adult rats regenerate from intrinsically different satellite cells. The Journal of Physiology. 2005 February 1, 2005;562(3):847-57.
[50] WANG XN, WILLIAMS TJ, McKENNA MJ, LI JL, FRASER SF, SIDE EA, et al. Skeletal Muscle Oxidative Capacity, Fiber Type, and Metabolites after Lung Transplantation. American Journal of Respiratory and Critical Care Medicine. 1999 July 1, 1999;160(1):57-63.

Figures:

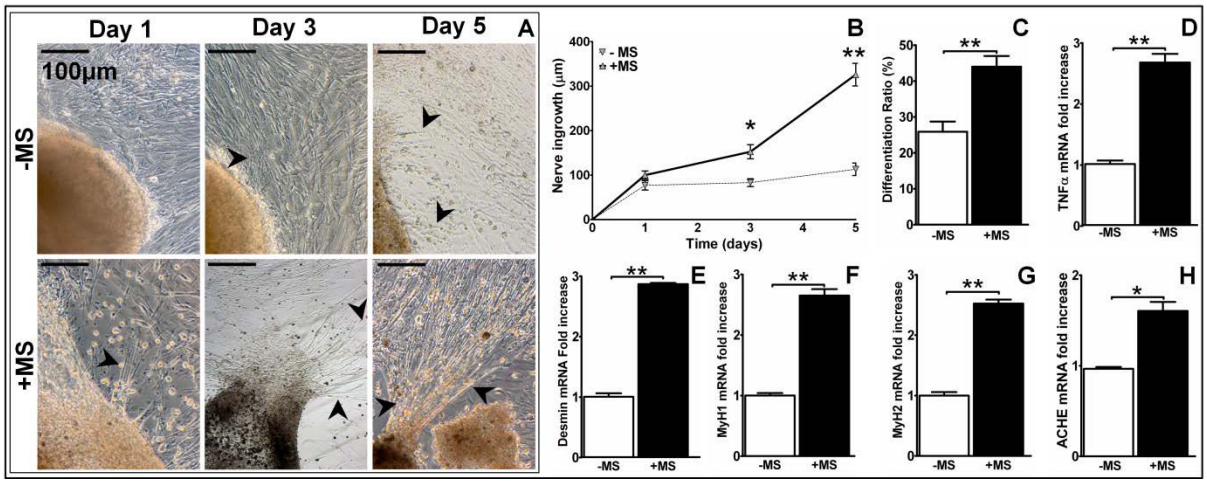

Figure 1 – **Magnetic Stimulation (MS) induces nerve ingrowth, muscle differentiation and triggers synapses**. We have submitted nerve-muscle co-cultures to MS and observed (A) the growth of neurites (black arrow heads) after each stimulation. (B) . After MS treatment, the neurites have grown significantly faster, with triple the length at 5 days of MS when compared to untreated control. Bold line on the plot represents MS treated samples and dashed line displays control unstimulated sample values. (C) Measurements of the fusion rate of newly formed myotubes demonstrated that MS increase the differentiation rate of myoblasts *in vitro*. To analyze the molecular expression of the co-cultures after MS treatment, RNA was isolated on day 5 of MS and compared to untreated control. (D) TNFα cellular expression increased up to 3-fold, confirming the presence of cell-signaling to induce myoblast differentiation. (E, F, G) Muscle specific markers as Desmin, Myosin Heavy Chain (MyH) 1 and 2 at least doubled their expression after MS treatment. (H) At the same time, the expression of Acetylcholine esterase (ACHE) nearly doubled (1.71±0.15) after MS stimulation. +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (*$p<0.05$, **$p<0.001$)

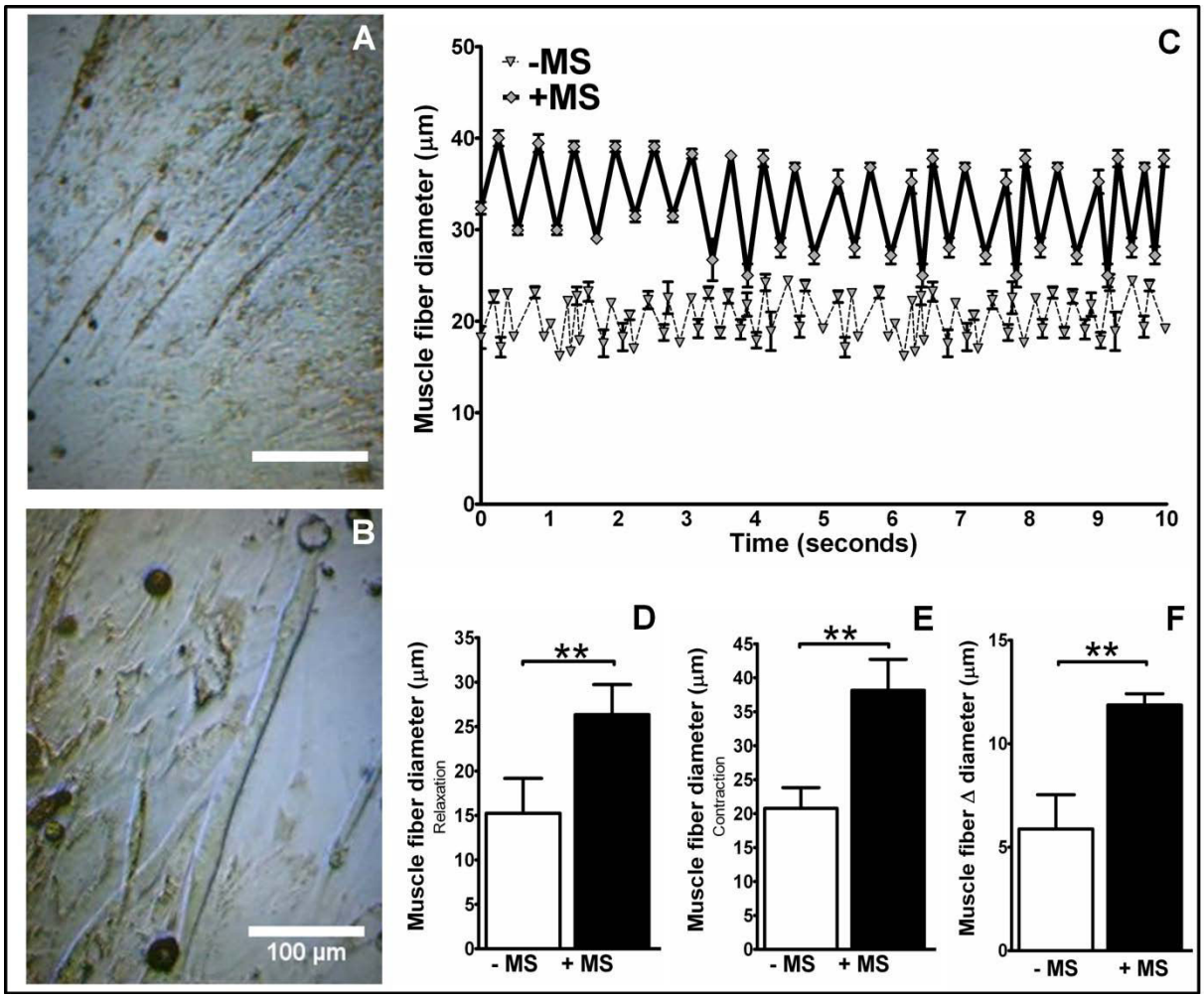

Figure 2 – **Contractile function of nerve-muscle co-cultures improves *in vitro* after MS treatment.** To assess contractile function of myotubes *in vitro* random fields of the Petri dishes were filmed (n=20) and their size and diameter at contraction and relaxation were measured. Snapshots of the relaxation state of (A) unstimulated and (B) MS stimulated co-cultures demonstrated the evident size and diameter differences of the two myofibers groups. (C) Measurements of two representative groups of fibers demonstrated that unstimulated fibers (dashed) display a fibrillation type of contraction, while MS treated (bold) fibers contract in a compassed rhythm. Separated measurements of relaxed (D) and contracting (E) fibers demonstrated an evident 2-fold increase of myotubes diameter. (F) The contractile delta, demonstrating the intensity of twitches was also more than doubled (control 5.88±0.28 and MS treated 11.87±0.54). +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (*p<0.05, **p<0.001)

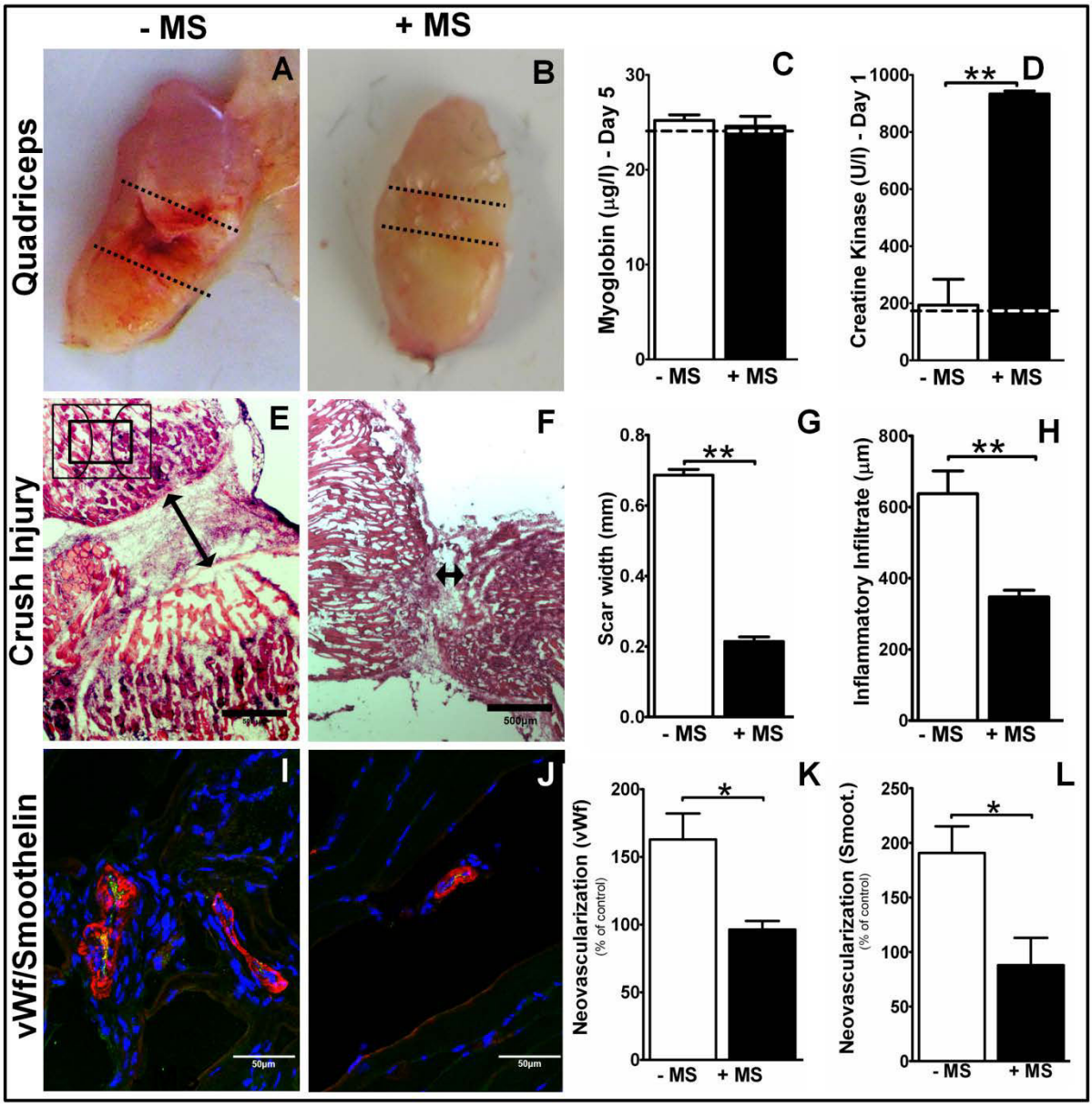

Figure 3 – **MS causes no systemic damage and accelerates regeneration process showing reduced scar width and inflammatory infiltrate.** Quadriceps was retrieved and blood samples were collected at time of sacrifice. (A, B) Quadriceps injury was smaller after MS treatment. (C) Levels of systemic myoglobin were comparable with normal parameters throughout the MS stimulation experiments. (D) Conversely, the systemic values of Creatine Kinase increased up to 5-fold. (E, F, G) Hematoxilin and Eosin stained sections demonstrated that the crush injury scar was significantly reduced after 5 days of MS treatment. This occurred by a replacement of damaged area with newly formed myotubes with central nuclei. (H) Simultaneously, the extension of the inflammatory infiltrate (presence of lymphocyte and macrophages) reduced more than a third. (I, J) Additionally, the formation of new vessels induced by inflammation was reduced already on day 5 after in MS treated samples. Staining were smoothelin/Cy3 (red), von Willebrand factor/FITC (green) and DAPI (blue). (K, L) While, control samples still displayed at least 150% higher number of microvessels, when compared to intact quadriceps, MS treated samples were demonstrating values similar to unscathed tissue. +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (*p<0.05, **p<0.001)

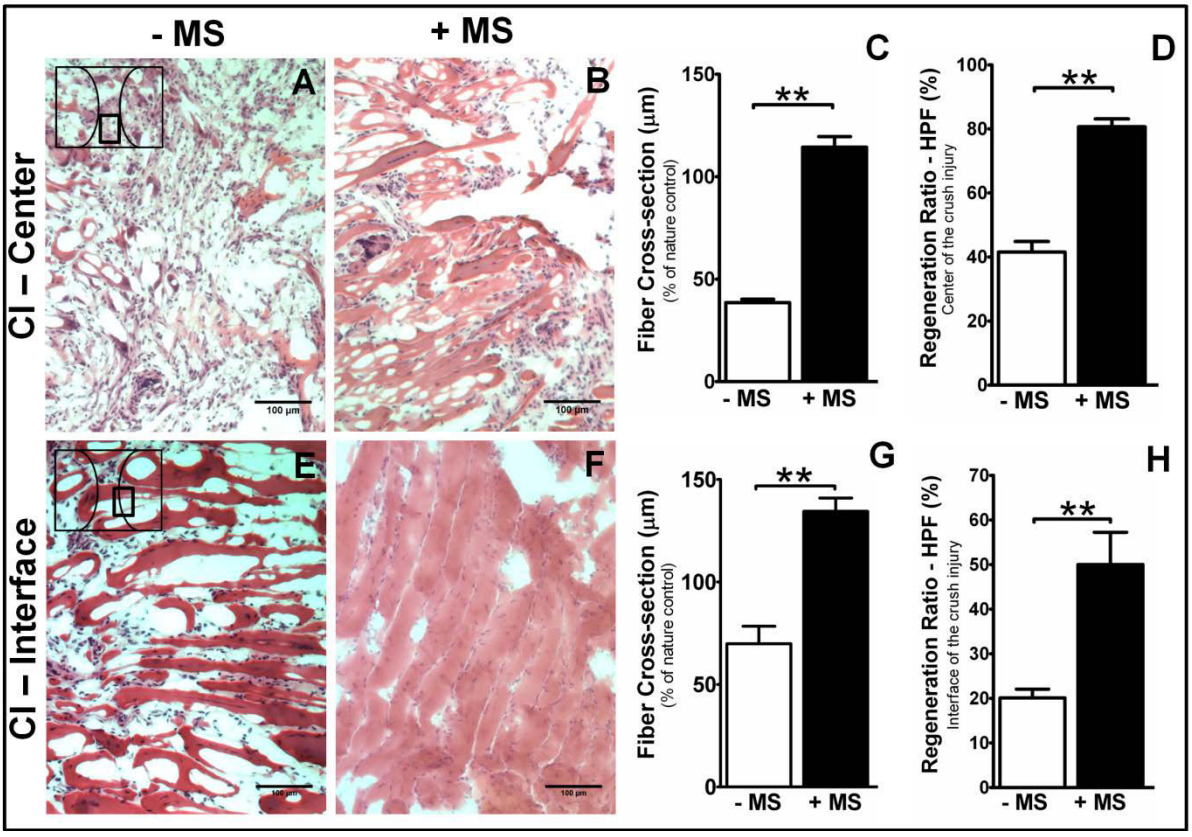

Figure 4 – **MS avoids post-trauma muscle atrophy, boosts muscle turn-over and induces hypertrophy of injury interface.** The isolated effect of MS on the injury site and on the injury interface was analyzed. (A, B, C) MS induced hypertrophy and doubled fiber cross-sections diameter. (D) This effect was associated with a remarkable increase in the regeneration ratio. (E, F) Similarly, in the injury interface the inflammatory infiltrate was reduced and (G) the fiber cross sections were again hypertrophic, with fiber cross sections around 40% bigger than in nature and MS untrained quadriceps. (H) Regeneration ratio, observed by the percentage of myofibers with central nuclei, was almost 3 times higher in stimulated samples. +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (**p<0.001)

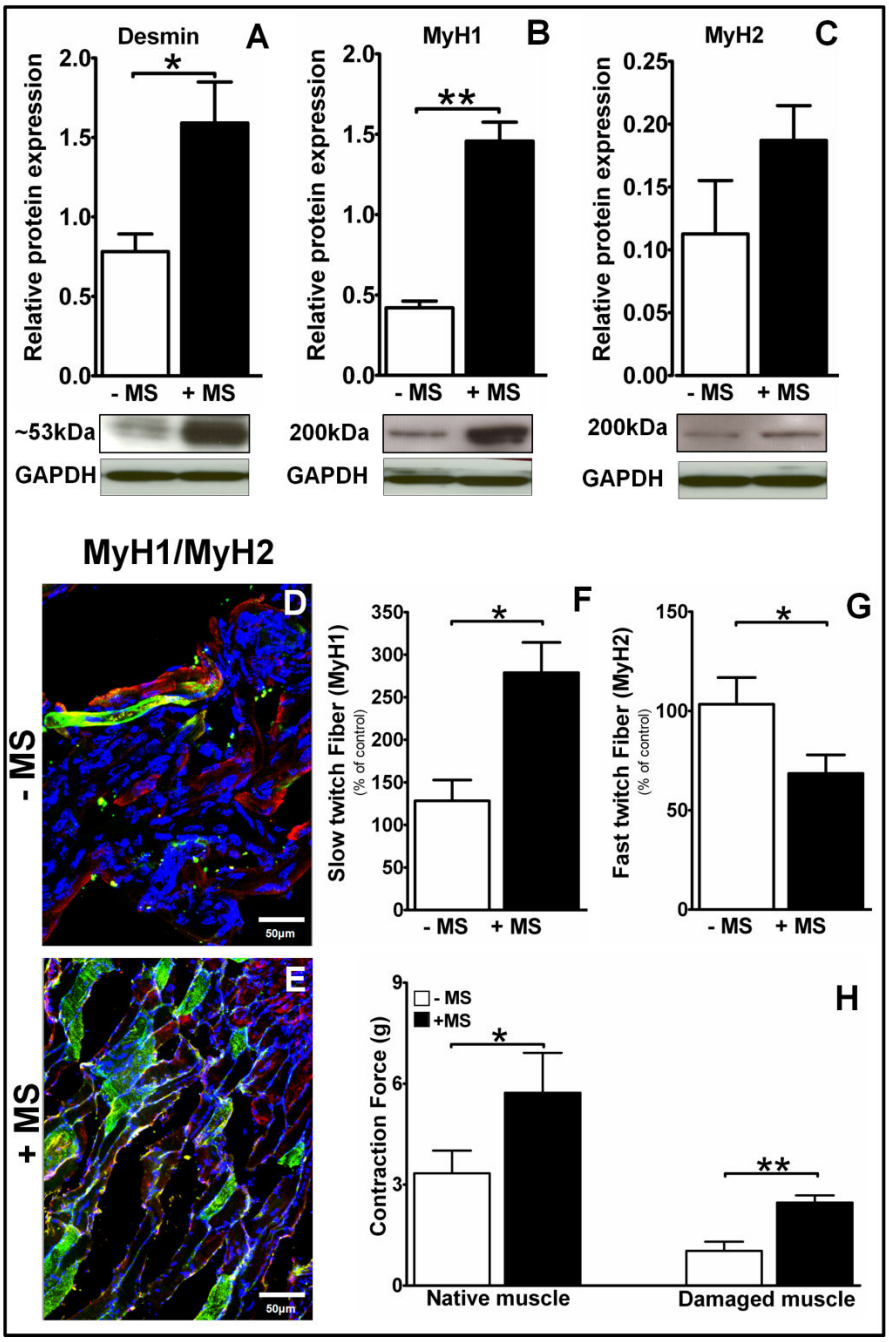

Figure 5 – **MS induces muscle type switch to slow twitch fibers and recovers muscle contractile strength after trauma** – After crush injury and MS treatment quadriceps was retrieved at time of sacrifice. Samples were shock frozen either for Western blot or histology. We found that muscle protein turn-over is increased after MS treatment. (A) Desmin nearly doubled its expression levels and (B) a specific increase of MyH type 1 could be detected. (C) No significant difference of MyH type 2 could be found on the WB. (D, E, F) When compared to intact control quadriceps, no fiber type change was found on injured muscle without MS stimulation. Staining was done with anti-myosin-heavy-chain-slow-twitch/FITC-anti-mouse-IgM (green), anti-myosin-heavy-chain-fast-twitch/Cy3-anti-mouse-IgG (red) and DAPI (blue). (E) On the other hand, a shift to fiber type 1 was verified in MS treated samples. (F) MyH type 1 expression was up to 3-fold higher in MS treated samples than in native muscle. (G) An expected MyH type 2 compensation decrease was detected. (H) Contractile function of muscle after exposition to MS treatment measured with a myograph. +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (*p<0.05, **p<0.001)

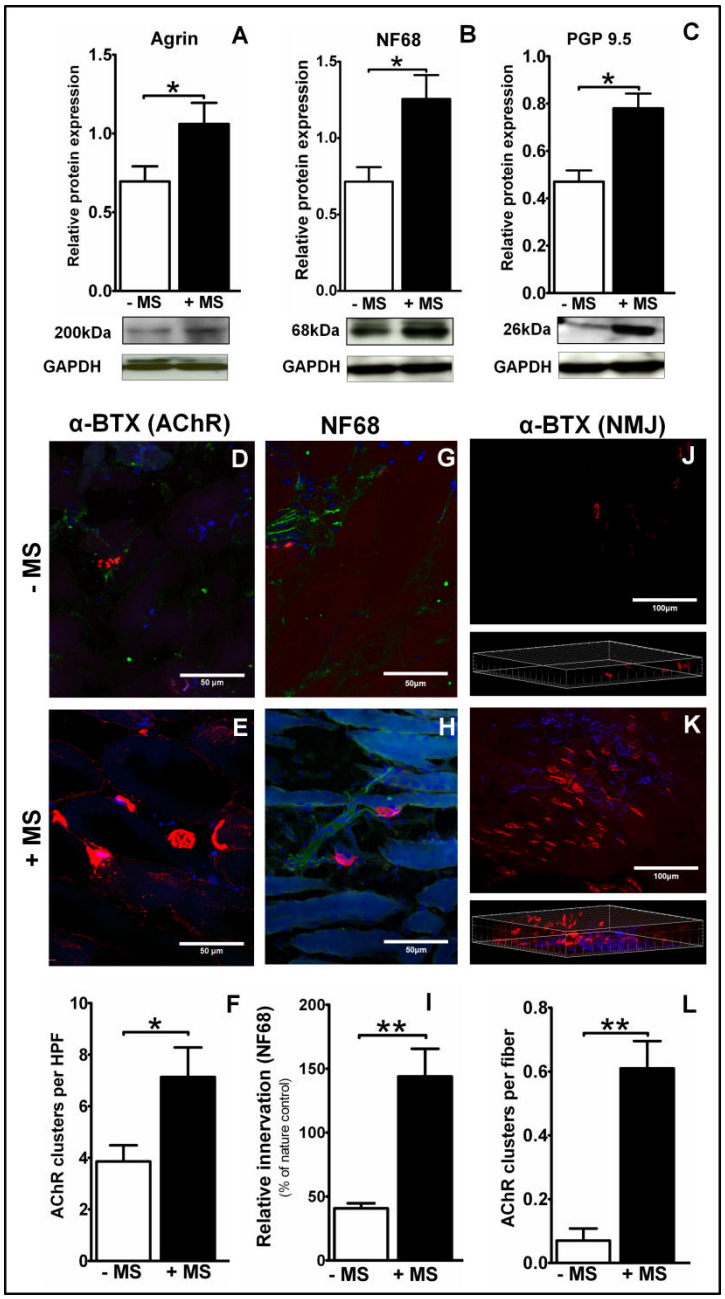

Figure 6 – **MS intensifies muscle-nerve cross-talk, increases nerve ingrowth and promotes AChR clustering.** Analyses of the nerve component within the injured muscle were performed by western blot (WB) and immunostaining. (A) Expression of Agrin was increased, (B, C) together with the nerve specific neurofilament 68 (NF68) and the PGP9.5-neuronal marker. (D, E, F) The study of the NMJ by staining of Acetylcholine receptor (AChR) with α-bungarotoxin (red) demonstrated that NMJ are more clustered and in significant higher density after MS. (G,H) Immunostaining of 10 µm tissue slices confirmed WB results, demonstrating an increased number of nerves inside of the tissue after MS. Staining was performed with neurofilament 68/488-anti-mouse-IGG (green), α-bungarotoxin (red) and DAPI (blue). (I) Surprisingly, the amount of nerves detected after MS was about 50% higher compared to normal tissue. (J, K) An analysis of the entire injury area digested with collagenase demonstrated the total ratio of AChR cluster per muscle fiber. (L) The total number of cluster per fiber was 4-fold higher in MS treated samples, demonstrating the positive effect of MS in reorganizing the neuromuscular junction after trauma. +MS indicates the presence of magnetic stimulation treatment, whereas -MS represent the unstimulated controls (*p<0.05, **p<0.001)

ANNEX I

Magnetic stimulation supports muscle regeneration after stem cell injection by boosting muscular metabolism and stimulating nerve ingrowth

The use of muscle precursor cells (MPCs) for the treatment of urinary incontinence through implantation in the urinary sphincter of pigs and dogs has been described[1, 2]. However, long term permanence and function of engrafts need to be established for an effective clinical application. Supporting the settling of transplanted cells and function of the newly formed tissue represents a cutting edge approach in tissue engineering. Initial clinical trials have evaluated this treatment option for the rehabilitation of pelvic floor dysfunction in women with urge-incontinence, stress incontinence and mixed incontinence and in men with post-prostatectomy early incontinence with promising results[3-6]. In this study we will investigate the mechanisms and applicability of magnetic stimulation (MS) to improve the muscular function of implanted MPC. Therefore, we have implanted MPCs into a site of acute muscle injury, exposed it to magnetic stimulation and evaluated muscle resistance of the engineered muscle by analyzing the expression of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1α). We test if magnetic stimulation is capable of eliciting the metabolism of muscle cells by up-regulating PGC-1α. This coactivator increases proportionally to the amount of exercise in a muscle[7, 8] and protects skeletal muscle from atrophy[9]. We implanted muscle cells into an area of induced muscle injury, submitted the animals to 5 days of MS (BioCon2000, 20 min, 50 Hz) and investigated the presence of synapses, clustering of acetylcholine receptors (AChRs) and muscular metabolic adaptations by immunohistochemistry, RTPCR and WB. MS increased stem cell differentiation *in vitro* and *in vivo* ($p<0.001$). Synapses frequency ($p=0.01$) and cluster of acetylcholine receptors (AChR) doubled under NMS treatment ($p=0.003$). Muscle metabolism and reconstruction were enhanced by the NMS therapy ($p<0.001$). Nerve ingrowth and branching was stimulated

($p<0.05$) and Agrin production was boosted ($p<0.05$). NMS significantly improved cell integration and distribution into the regenerating muscle ($p<0.001$) and increased the number of fibres formed by the implanted MPCs. NMS improves differentiation of implanted MPCs into functional muscle, by improving synapses, and boosting nerve ingrown and AChR clustering.

REFERENCES

1. Holzer, N. *et al.* Autologous transplantation of porcine myogenic precursor cells in skeletal muscle. *Neuromuscular Disorders* **15**, 237-244 (2005).
2. Eberli, D., Aboushwareb, T., Soker, S., Yoo, J.J. & Atala, A. Muscle Precursor Cells for the Restoration of Irreversibly Damaged Sphincter Function. *Cell Transplant* (2012).
3. Teruhiko Yokoyama, O.F., Jun Nishiguchi, Kunihiro Nozaki, Hiroyuki Nose, Miyabi Inoue, Hideo Ozawa, Hiromi Kumon Extracorporeal magnetic innervation treatment for urinary incontinence. *International Journal of Urology* **11**, 602-606 (2004).
4. Yokoyama, T.N., Jun; Watanabe, Toyohiko; Nose, Hiroyuki; Nozaki, Kunihiro; Fujita, Osamu; Inoue, Miyabi; Kumon, Hiromi Comparative study of effects of extracorporeal magnetic innervation versus electrical stimulation for urinary incontinence after radical prostatectomy. *Urology* **63**, 264-267 (2004).
5. But, I., Faganelj, M. & Sostaric, A. FUNCTIONAL MAGNETIC STIMULATION FOR MIXED URINARY INCONTINENCE. *The Journal of Urology* **173**, 1644-1646 (2005).
6. Ishikawa, N. *et al.* in Engineering in Medicine and Biology Society, 1997. Proceedings of the 19th Annual International Conference of the IEEE, Vol. 5 2096-2099 vol.20951997).
7. Mathai, A.S., Bonen, A., Benton, C.R., Robinson, D.L. & Graham, T.E. Rapid exercise-induced changes in PGC-1{alpha} mRNA and protein in human skeletal muscle. *J Appl Physiol* **105**, 1098-1105 (2008).
8. Pilegaard, H., Saltin, B. & Neufer, P.D. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* **546**, 851-858 (2003).

9. Sandri, M. *et al.* PGC-1 alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. *Proceedings of the National Academy of Sciences* **103**, 16260-16265 (2006).

FIGURE

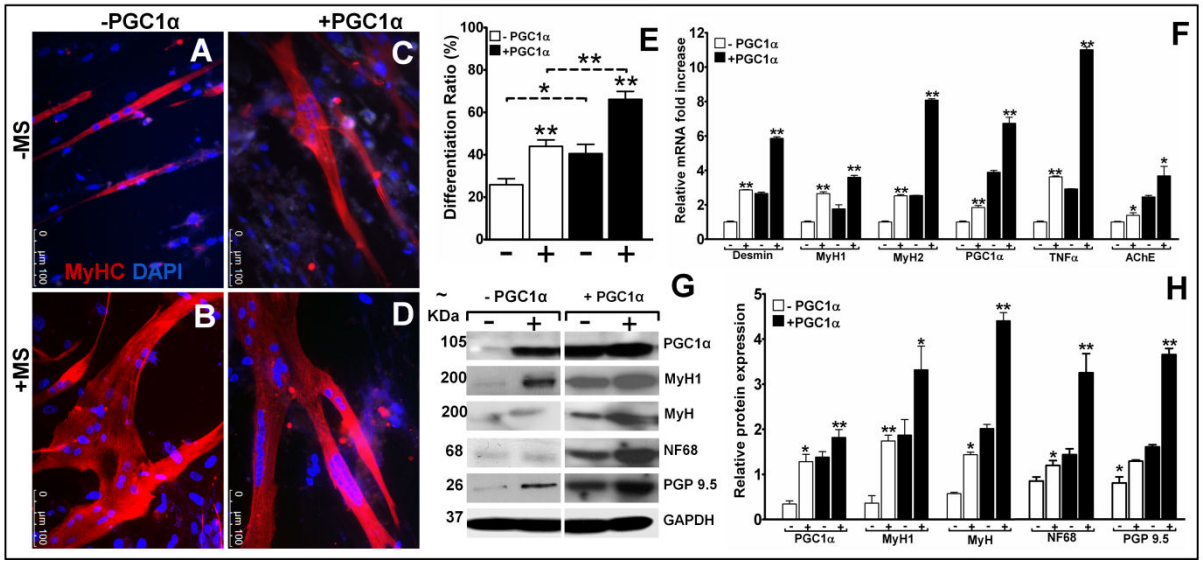

Figure 1 - Magnetic stimulation effects on MPCs *in vitro*. Co-cultures of MPCs and spinal cords slices were submitted to alternating magnetic pulses for 5 days (20 min, 50 Hz). (A, B, E) MS increased fiber formation *in vitro* after 5 days of stimulation. (C, D, E) Cells transfected with PGC1α adenovirus was sufficient to increase cell differentiation and was boosted with the MS treatment. (F) Measurements of mRNA demonstrated that muscle factors like desmin, myosin slow- (MyH1) and fast-twitch more than double after MS. Likewise, the levels of PGC1α expression double with MS even in already transfected cells. TNFα increase expression, supporting the results of the differentiation rate assay. Yet, the increase of synapses in both cell groups could be verified by Acetyl cholinesterase expression. (G, H) Protein semi-quantitative analyses with western blot confirmed the increased expression of PGC1α in MS stimulated samples to similar values of PGC1α-transfected cells. Muscle (general and slow twitch Myosin heavy chain –MyH and MyH1) and nerve (neurofilament 68 – NF68 and PGP9.5) components demonstrated MS capacity of boosting muscle formation and nerve ingrowth. *$p<0.001$, **$p<0.05$

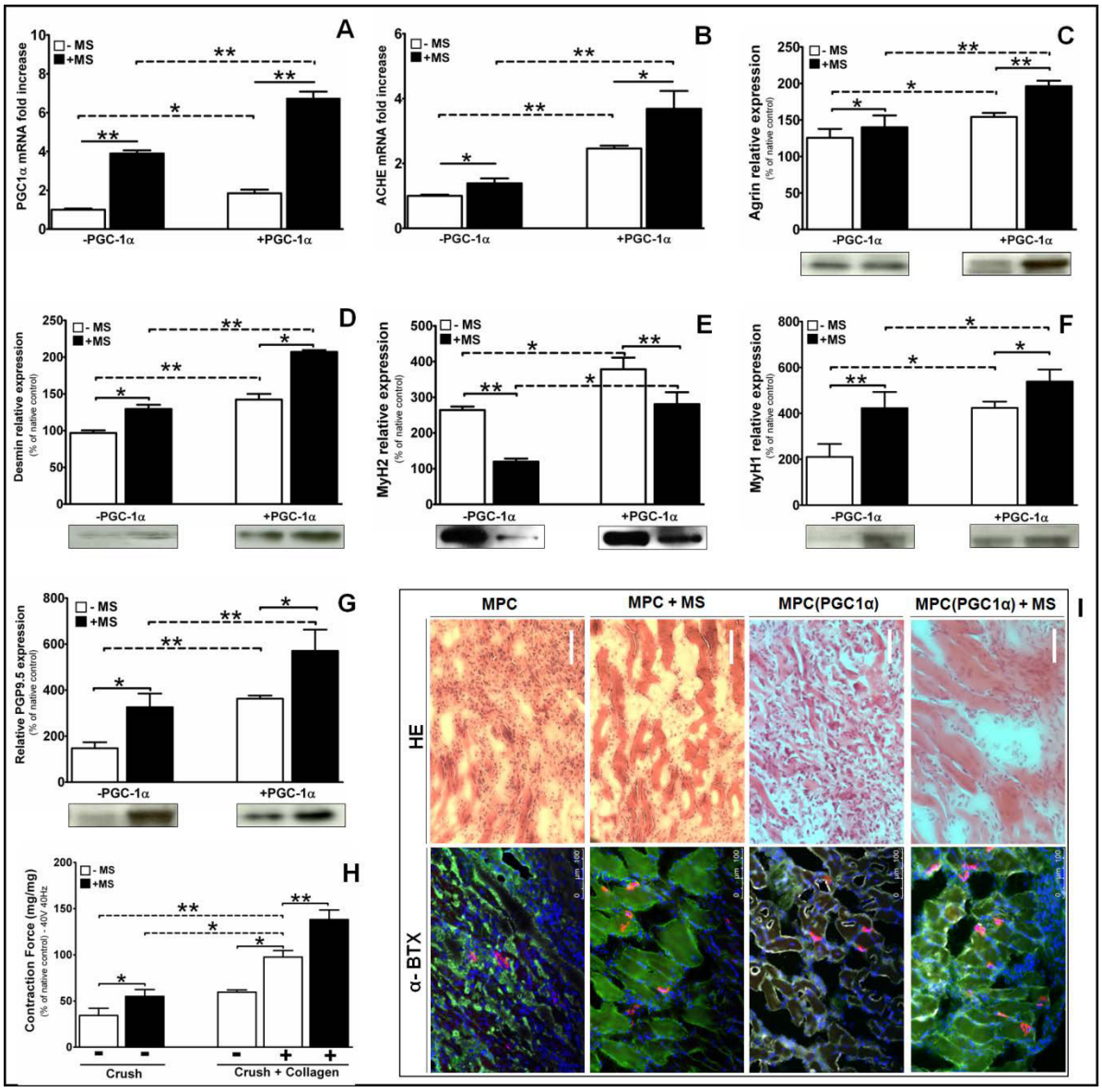

Figure 1 - Magnetic stimulation effects on MPCs *in vivo*. We have transfected muscle precursor cells (MPCs) with GFP or PGC1α adenovirus and injected 10 million cells in a collagen carrier into crushed quadriceps muscle. Half of the animals were submitted to alternating magnetic pulses for 5 days (20 min, 50 Hz). (A) The expression level of PGC1α were confirmed by mRNA expression and doubled after MS treatment. (B) The increase of synapses was demonstrated by mRNA expression of acetyl cholinesterase (ACHE) after MPC injection and MS stimulation demonstrating that the regeneration process was increased. (C) The expression of Agrin significantly increased after injection of MPCs and MS stimulations. (D) Expression of desmin was also significantly increased by the presence of MPC and MS. (E, F) Fiber type switch from fast- to slow-twitch was observed after MS and MPC. (G) Ingrowth of nerves into the newly implanted and MS stimulated tissue could be detected by the increase of PGP 9.5. (H) Muscle contractile function was also boosted by the treatment with MPC and MS (- no cell injection, + MPCs injection in collagen carrier). (I) Integration of MPCs could be detected by the accumulation of GFP into the new formed myotubes. *p<0.001, **p<0.05

ACKNOWLEDGEMENTS

I want to thank the one that held my hand, saw my tears and gave me a hug in days when things were not working well. I want to thank the one that shared my joy and celebrated with me every victory. You are the one that always made me believe that a can go beyond my limitations. You are simply the One, my companion, my best friend, my husband, my Kai. Thank you for being there for me!

I want to thank the joy of my days that made the completion of this thesis a pleasant adventure… my sunshine, baby girl, my Lorie.

You were the ones that knew me from the first day of life, who taught me how to walk and accompanied every step that followed the first one… my friends, my fans, my parents; Julia and Josimar. Thank you so much! (Vocês foram aqueles que me conheceram desde o meu primeiro dia de vida, vocês me ensinaram a andar e acompanharam cada passo que seguiu o primeiro de todos… meus amigos, meus fãs, meus pais, Julia e Josimar. Obrigada por tudo!)

I would like to thank the one that believe in me from the beginning, saw in me potential and decided to invest in it, my supervisor and supporter Daniel Eberli.

I would like to thank the ones that believed in the idea of this project from the very beginning and gave me the support and advice to make it scientific sound… Prof. Sulser, Prof. Besckei, Prof. Handschin and Dr. Ferrari.

You shared with me everyday life in the lab, helped me to understand new techniques and laughed with me about my personal and common mistakes. Thank you so much that you were real friends in this journey… my companions, my buddies, my colleagues, Lukas, Souzan, Fahd, Fatma, Maya, Sarah, Damina, Mathias, Deana, Ria, etc..

You shared with me everyday smiles and troubles. You prayed for me and encouraged me in each step. You are a treasure in my life everyday… more than friends; you are brothers and sisters, Monia, Walker, Glauca, Thatiana, Kelly, Lukas, Tirsa, etc..

Dedication:

To the one that showed me that every straggle to do what is right is worthy it; that helped me to believe in my ability to grow and learn; that gave me the creativity to develop new ideas, the courage to apply these ideas into new projects and the strength to face the hard days with a hope of a better tomorrow… my architect, my provider, my saviour, my father, my Jesus, my God.

CURRICULUM VITAE

*Meline Nogueira Lucena Stölting**

Personal Data

Name: Meline Nogueira Lucena Stölting, MD.
Date of Birth: 30.07.1982
Nationality: Brazilian
Marital Status: Married, 1 daughter
Office: Urologische Klinik / Universitätsspital Zürich
Frauenklinikstr. 10. NORD I O42. CH-8091 Zürich
Home: Pestalozzistrasse 7 CH-4600 Olten
Office: +41 44 255 9301
Privat: +41 43 499 0187
Mobile: +41 76 570 5862
Email: meline.stoelting@usz.ch
melinestoelting@yahoo.com

Education

2012 – present	Post-Doc at the Urology Clinic, University of Zürich, Switzerland.
2009 – 2012	MD-PhD at the University of Zürich, Switzerland.
2001 – 2007	Diploma as medical doctor Federal University of Amazonia, Manaus, Brazil, rated 93/100 (Working hours= 7365) Basic studies 2001-2003, graded 85%/100%, UFAM Main studies 2003 – 2006, graded 81%/100%, UFAM Hospital Practice 2006 – 2007, graded 93%/100%, UFAM (More information about extracurricular activities on the topics *Clinical Experience, Teaching Training and Research experience*).
1997 – 2000	Graduation on High School and as lab technician with 85/100 from the *Escola Técnica Federal do Amazonas – ETFAM* (Federal Amazonian Technical School), Manaus, Brazil. (Working hours = 5197)

Dissertation

MD (Dr. Med.)	Federal University of Amazonia, Manaus, Brazil. Supervisor: Luiz Ferreira, MD PhD. Title: *The use of polymerase chain reaction to confirm diagnosis in skin biopsies consistent with American tegumentary leishmaniasis at histopathology* (http://www.ncbi.nlm.nih.gov/pubmed/22147028)
PhD (Dr. sc. nat.)	University of Zürich. Supervisors: Attila Becskei, PhD and Daniel Eberli, MD PhD PD. Title: *Adult Muscle Progenitor Cells for Clinical Applications: Function, Safety and Interactions.*

Clinical Experience (Clinical and Surgical Practice)

2008	***Urologic Clinic of the University Hospital Zürich*** (UniversitätsSpital Zürich), Switzerland. Post: Gastärztin (Invited Doctor – 100%). Duties and Responsibilities: Ambulatory and ward patients follow-up.
2007	***Public Health Medicine*** at Coari. Post: Familiar Assistant doctor. Duties and Responsibilities: Primary Medical ambulatory Assistance.
2007	***Clinical Emergencies (ACLS training)*** on several Emergency rooms in Manaus, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Basic Life Support and Emergency Room active work.
2006 – 2007	***Surgical Emergencies (ATLS training)*** at the General Emergence Hospital, Manaus, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Surgical Emergence, Trauma, Life Support.
2006 – 2007	***Gynecologic and Obstetric Emergencies training*** at the Maternity Ana Braga, Manaus, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Emergences, Prenatal and Parturition Assistance.
2006	***Pediatric Emergencies Training*** Room on the Children Hospital, Manaus, Amazonas, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Neonatal care, general children care.

**Premarital name: Meline Nogueira Barbosa de Lucena*

2005 - 2006 ***Basic Life Support and Clinical Emergence training*** at the Tropical Medicine Foundation of Amazonas, Manaus, Brazil. Post: Doctor Assistant. Duties and Responsibilities: Diagnostic and Treatment of Ambulant and Ward patients.

2005 **Intensive Care Unity** on the University Hospital Getulio Vargas, Manaus, Amazonas, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Monitoring critical patients.

2004 - 2005 **Clinical Emergence Room in Oncology** at the *Fundação de Controle de Oncologia (FCECON) do Amazonas*, Manaus, Amazonas, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Oncologic Emergencies and Pain Management.

2003 - 2004 **Surgery Emergency Training** at the Emergency Room SPA-Alvorada, Manaus, Brazil. Post: Intern (Unterassistent). Duties and Responsibilities: Surgical Emergence, Trauma, Life Support.

Summary of Procedures and Skills

	Assistant	**Independent**
Obstetrics & Gynaecology	Hysterectomy, Tubal ligation, Perineoplasty, Caesarean operation, etc.	Obstetric and Gynaecology Clinical Exam; Prenatal, Parturition assistance, Plastic perineal sutures, Papanicolaou sampling, IUD insertion, STD diagnostic and treatment (including Nitrogen application on genital warts). Basic Obstetric Ultrasonography.
General Surgery	Herniorrhaphy, appendicectomy, Colescistectomy, Emergence surgery, intestinal sutures, Splenectomy, Pericardiocentese.	Surgical and Emergency Clinical Exam; Central and peripheral venous catheterization; Thoracocentesis; Paracentesis; Surgical debridement; Cutanous sutures (plastic or haemostatic); venous dissection; emergencies small surgeries (Traqueotomy, Thoracotomy); Basic Surgical and Urological Ultrasonography; Biopsies
General Clinic	Specialized Clinical Care	Resuscitation Protocol (according with ACLS); Intubation, Arterial punction (radial and Femoral), bladder catheterization. Management of AIDS, ophidian accidents, malaria, Leishmaniasis, Pneumonia, Desnutrition, Diarrheic Syndromes, among others.

Research Experience

2009 – Present PhD Student at the Laboratory for Tissue Engineering – Urologic Clinic of the University Hospital Zürich (UniversitätsSpital Zürich), Switzerland. Project: ***Adult Muscle Progenitor Cells for Clinical Applications: Function, Safety and Interactions.***

2008 – 2009 Research Fellow (Wissenschaft Mitarbeiterin) at the Laboratory for Tissue Engineering – Urologic Clinic of the University Hospital Zürich (UniversitätsSpital Zürich), Switzerland (100%). Project: ***Interaction between Adult Stem Cells (ASC) and preexisting Cancer in Tissue Engineering***

2008 Research Assistant at the Molecular and Evolutionary Biology – Zoological Museum at the University of Zürich-Irchel.

2005 – 2007 Research assistant at the Pathology Department in the *Fundação de Medicina Tropical do Amazonas* – **FMTAM** (Tropical Medicine Foundation of Amazonia), Manaus, Amazonas, Brazil. Project: ***Diagnosis of Tegumentary Leishmaniasis in Paraffin Embedded Samples via Polymerase Chain Reaction***. Abstract: 1

2003 - 2005 Research assistant at the Dermathology Department (Leishmaniose Laboratory) in the *Fundação de Medicina Tropical do Amazonas* – **FMTAM** (Tropical Medicine Foundation of Amazonia), Manaus, Amazonas, Brazil. Project: ***Isolation and Characterization of Leishmania Species from reinfected patients with Tegumentary Leishmaniasis***. Publications: 2

2002 – 2003 Member of the Special Training Program for Medical Students (PET).
Projects: ***(a) Evaluation of High Arterial Blood Pressure and its Risk Factors on a Riverine population, Terra Santa. (b) Coproparasitologic Screen of Children from a Riverine Population at Piraruacá Lake, Terra Santa – PA. (c) Evaluation of Education Quality in Medical Studies in the UFAM: a graduation analyses.*** Publications: 4, 5, 6, 8, 9, 10, 11

2002 – 2003 Research Assistant at the Molecular Biology Laboratory in the *Instituto Nacional de Pesquisas da Amazonia* – ***INPA*** (National Amazonian Research Institute), Manaus, Amazonas, Brazil. Project: ***Phylogeography of Hypopygus lepturus (Rhamphichthyidea: Gymnotiformes): Testing the Rio Negro as a Dispersal Barrier.*** Publications: 13, 14

Grants, Honors and Awards

2012 **Research Grant – Novartis Stiftung, Switzerland** for the research project: "Improving human muscle engineering by PGC-1α expression and molecular imaging using positron emission tomography (PET)" (CHF 60'000.00).

2012 **Research Grant – Promedica/UBS, Switzerland** for the research project: "Improving human muscle engineering by PGC-1α expression and molecular imaging using positron emission tomography (PET)" (CHF 165'800.00).

2012 **Internal Grant Matching Funds, Switzerland** for the research project: "Improving human muscle engineering by PGC-1α expression and molecular imaging using positron emission tomography (PET)" (CHF 80'000.00).

2012 **Prize: Best Poster** for the research project: "*In vivo* Electromagnetic stimulation supports muscle regeneration after stem cell injection by boosting muscular metabolism and stimulating nerve ingrowth". Annual Meeting of the American Urological Association, Atlanta 2012

2011 **Prize: Best Poster of Session** for the research project: "Muscle Precursor Cells are safe for the treatment of urinary incontinence after surgery for prostate cancer". Annual Meeting of the European Assocciation of Urology, Vienna 2011

2011 **Participation on Sinergia Research Grant from SNF, Switzerland** for the research project: "Improving human muscle engineering by PGC-1α expression and molecular imaging using positron emission tomography (PET)" (CHF 1'300'000.00).

2009 **Research Grant from Forschungskredit, Switzerland** for the research project: "Adult Muscle Progenitor Cells for Clinical Applications: Function, Safety and Interactions" (CHF 134'000.00).

2009 **Research Grant from EMDO Stiftung, Switzerland** for the research project: "Adult Muscle Progenitor Cells for Clinical Applications: Function, Safety and Interactions" (CHF 30'000.00).

2009 Recognized by the **University of Zürich** and approved as a member of the prestigious MD-PhD Program MNF/MF, University of Zürich.

2008 Recognized by the **University of Zürich** and selected for Presentation at the **Researchers' Nights**, September 2008: Stem Cells and Urinary Incontinence.

2008 **Research Grant from Abbott, Switzerland** for the research project: "Interaction between Adult Stem Cells (ASC) and preexisting Cancer in Tissue Engineering"(CHF 5'000.00).

2008 Grant from **Hartmann Müller-Stiftung für Medizinische Forschung,** for the project: "Interaction between Adult Stem Cells (ASC) and preexisting Cancer in Tissue Engineering". (CHF 15'000.00)

2005 Grant from the Research Support Foundation of Amazonas State (FAPEAM), for the project: "Diagnosis of Tegumentary Leishmaniasis in Paraffin Embedded Samples via Polymerase Chain Reaction", total of R$ 15'000,00.

2003 Grant from the Research Support Foundation of Amazonas State (FAPEAM), for the project: "Isolation and Characterization of Leishmania Species from reinfected patients with Tegumentary Leishmaniasis" , total of R$ 14'000,00.

2003 Award for the Social work done at the city of Terra Santa, Brazil.

2002 Grant from the National Counsel of Technological and Scientific Development (CNPq – Brazil). For the project: "Preliminary mitochondrial DNA diversity analyses of *Hypopygus lepturus* (Gymnotiformes, Rhamphichthyidae) from the middle Rio Negro", total of R$ 7'000,00.

1998 Award for the Best Technical Research Project: "Breu: uma nova fonte de Aroma da Amazônia"

Congress and Courses Participation

2012 Oral Presentation in the EAU Conference (Paris, France): "*In vivo* Electromagnetic stimulation supports muscle regeneration after stem cell injection by boosting muscular metabolism and stimulating nerve ingrowth".

2012 Oral Presentation in the EAU Conference (Paris, France): "Noninvasive Electromagnetic Stimulation for stress urinary incontinence improves regeneration of skeletal muscle, increases nerve ingrowth and acetylcholine receptor clustering".

2010 Oral Presentation in the TERMIS NA 2010 Conference (Orlando, Florida): "Impact of patient age or gender on bioengineering of functional muscle tissue using Muscle Precursor Cells".

2010 Oral Presentation in the TERMIS NA 2010 Conference (Orlando, Florida): "Muscle Precursor Cells inhibit tumor growth upon secretion of TNF alpha in vitro".

2010 Co-chair of the section Mesenchymal Stromal Cells: Characterization and Expansion of the conference TERMIS EU 2010, Galway, Irland

2009 "Introductory course in Laboratory Animal Science" – LTK/University of Zürich, Zürich, Switzerland.

2009 "GCP – Good Clinical Practice – Module 1" – ZKF/University of Zürich, Zürich, Switzerland.

2009 "GCP – Good Clinical Practice – Module 2" – ZKF/University of Zürich, Zürich, Switzerland.

2009 "Targets for Cancer Prevention and Therapy" – USZ/University of Zürich, Zürich, Switzerland.

2009 "1. Interdisziplinären Uro-Onkologischen Symposium" – USZ/University of Zürich, Zürich, Switzerland.

2008 Scientific Exposition – "Nacht der Forschung" – ETH/University of Zürich, Zürich, Switzerland. ***Exposition of the stem cell therapy for Urinary Incontinence.***

2008 Symposium – Mouse Models for Human Diseases, Zürich, Switzerland

2008 95th Annual National Congress of Surgery (Jahreskongress der Schweizerischen Gesellschaft für Chirurgie), Basel, Switzerland

2005 III UNIMED MANAUS Medical Congress at the Tropical Hotel Manaus, Brazil.

2004 II UNIMED MANAUS Medical Congress at the Tropical Eco-Resort Convention Center, Manaus, Brazil.

2004 Ciclo de Palestra: Cirurgia Otológica e Cirurgia Endoscópica Naso-Sinusal, Manaus, Brazil.
2003 XLI Congresso Brasileiro de Educação Médica, Florianopólis, Brazil.
2003 Simpósio de Infecção Hospitalar, Manaus, Brazil.

Teaching Experience

2012 Co-supervision of the PhD-Student: Deana Haralampieva from the ETH, Zürich, at the Laboratory for Urologic Tissue Engineering and Stem Cell Therapy, University Hospital Zürich.
2010 Supervision of the MD-Student: Ryan Khanna from the Northwestern University, Evanston, IL, at the Laboratory for Urologic Tissue Engineering and Stem Cell Therapy, USZ.
2006 Training in Teaching as Lecture Assistant (Tutor) in Epidemiology at the Public Healthy Department of the Federal University of Amazonas, making 141 Working hours
2005 - 2006 Training in Teaching on the "Liga Universitária de Clínica Médica" at the Federal University of Amazonas, realized between August 1st 2005 and July 31st of 2006, making 300 Working hours.
2005 - 2006 Training in Teaching on the "Liga Universitária de Cardiologia e Cirurgia Vascular" at the Federal University of Amazonas, realized between August 1st 2005 and July 31st of 2006, making 300 Working hours. Publication: 7, 8
2002 - 2004 Training in Research and Teaching on the Special Training Program (Programa Especial de Treinamento – PET) at the Federal University of Amazonas, making 720 Working hours
2002 Training in Teaching as Lecture Assistant (Tutor) in Human Anatomy at the Anatomy Department of the Federal University of Amazonas, making 141 Working hours

Social and Public Health Experience (Social Actions, Health Assistance to poor communities)

2007 Social Action on Prevention of Diseases and Planning of the Healthy Care on a median city on middle Amazon Forest, Coari. Realized between February 1st till April 13th, totalizing 720 h.
2004 Amazonas River and Piraruacá Comunities – Brazil, Healthy Assitance in association with IBAMA on the project: "***Manejo Sustentável de Quelônios nos Municípios de Terra Santa, Oriximiná/PA, Nhamundá e Parintins/AM – Pé de Pincha***". Realized between April 1st till July 31st of 2004, 300 h. Publication: 4, 5
2003 Health assistance and Social Action on poor communities into Amazonic Forest **– *An endemic area of Leishmaniasis Pau Rosa Community – Amazonia***.

Languages:

German (fluent)
Swiss German (Hörverständnis)
English (fluent and scientific)
Portuguese (native speaker)
Spanish (school level)

Technical Skills

DNA-extraction; PCR (Polymerase Chain Reaction); RTPCR; DNA Sequencing; AFLP- (Amplified Fragment Length Polymorphism) Analyses; FACS; Animal model experiments; Bacterial and Protozoan culture; cell isolation and culture (various types); preparation of slides for histological studies; Sterilization Methods; Electrophoreses; Isoenzyme analyses; Monoclonal Antibody testing and production; Western Blot; Immunohistochemistry, Fluorescence and light microscopy; Handling and production of Adenovirus and Plasmids; Organ Bath Methods, among others.
Computer literate on PC: MS Office, BioEdit and Sequencer, Adobe (Acrobat, Photoshop, Distiller), Flowjo, GraphPad, IMAGE J, IMARIS, Corel Draw, SPSS, EpiInfo, R-statistics; among others.

Referees

PD. MD. PhD. Daniel Eberli
Leitenderarzt Urologische Klinik
UniversitätsSpital Zürich
Email: daniel.eberli@usz.ch

Prof. MD. Tullio Sulser
Direktor der Urologische Klinik
UniversitätsSpital Zürich
Email: tullio.sulser@usz.ch

Prof. PhD Luiz Carlos Ferreira
Senior Prof. of the Pathology Department (UFAM)
Director of the Research Department (FMTAM)
Email: ferreira@fmt.am.gov.br

Prof. PhD. Dirceu Benedito Ferreira
Professor of the Pathology Department
Federal University of Amazonas (UFAM)
Email: dirceu@vivax.com.br

Publications

Book Champter

1. Fahd Azzabi; **Meline N. L. Stölting**; Daniel Eberli. *Cell Therapy for muscle regeneration.* Submitted to Regenerative Medicine and Tissue Engineering.

Published Reports (Peer reviewed Journals)

1. ANDRADE RV, MASSONE C, **LUCENA MNB**, TALHARI AC, TALHARI S, GUERRA JAO, FERREIRA LCL. *The use of polymerase chain reaction to confirm diagnosis in skin biopsies consistent with American tegumentary leishmaniasis at histopathology: a study of 90 cases.* An Bras Dermatol. 2011;86(5):892-6
2. **LUCENA, M. N. B**; COELHO, L. I. A. R. C; ALMEIDA, M. F.; PAES, M. G; FERREIRA, L. C. L; TALHARI, S.. *Isolamento e caracterização de cepas de pacientes portadores de leishmaniose tegumentar com reinfecção.* In: Resumos do XLI Congresso da Sociedade Brasileira de Medicina Tropical, 2005, Florianopolis-SC. Revista da Sociedade Brasileira de Medicina Tropical, 2005. v.38. P. 356-7
3. GUERRA, J. A .O.; COELHO, L. I. A. R. C; GUERRA, M.; BRÍGIDA, R. S., **LUCENA, M. N. B;** ALMEIDA, M. F.; PAES, Marcilene G.. *Reinfecção em Leishmaniose Cutânea – Registro de 210 casos atendidos na Fundação de Medicina Tropical do Amazonas.* 2005. Revista da Sociedade Brasileira de Medicina Tropical, 2004. P. 247-8

In print

4. **Meline N. L. Stölting**; Stefano Ferrari; Christoph Handschin; Attila Becskei; Tullio Sulser; Daniel Eberli. *Myoblasts inhibit tumor growth by paracrine secretion of TNF alpha.* Accepted in Journal of Urology.

In Preparation

5. **Meline N L Stölting**; Lukas J Hefermehl; Mathias Tremp; Fahd Azzabi; Remo Largo; Tullio Sulser; Daniel Eberli. *Impact of patient age or gender on bioengineering of functional muscle tissue using Muscle Precursor Cells.* Submit to Regenerative Medicine
6. Mathias Tremp; **Meline Stölting**; Lukas Hefermehl; Tullio Sulser; Daniel Eberli. *Contractile function of engineered muscle tissue depends on donor harvest location.* Submit to Muscle and Nerve
7. **Meline N. L. Stölting**; Christoph Handschin; Anne Sophie Arnold; Deana Haralampieva; Tullio Sulser; Daniel Eberli. *Magnetic Stimulation mimics the effect of exercise on skeletal muscle, improves regeneration of muscle tissue and boosts nerve ingrowth after injury.* Submit to Neurorehabilitation and Neural Repair
8. **Meline N. L. Stölting**; Christoph Handschin; Attila Becskei; Tullio Sulser; Daniel Eberli. *Magnetic Stimulation improves innervation and tissue formation after stem cell injection.* Submit to TERMIS

Abstracts and Posters

1. **Meline N L Stölting**; Lukas J Hefermehl; Mathias Tremp, Ryan Khanna, Fahd Azzabi, Tullio Sulser; Daniel Eberli. (2012) *Age and gender limitation for the bioengineering of contractile muscle tissue for human Muscle Precursor Cells (MPC).* In: 2012 World Congress of Tissue Engineering and Regenerative Medicine.
2. **Meline N L Stölting**; Christoph Handschin; Attila Becskei; Tullio Sulser; Daniel Eberli. *In vivo* Electromagnetic stimulation supports muscle regeneration after stem cell injection by boosting muscular metabolism and stimulating nerve ingrowth. In: 2012 Annual Meeting of the European Association of Urology.
3. **Meline N L Stölting**; Christoph Handschin; Attila Becskei; Tullio Sulser; Daniel Eberli. Noninvasive Electromagnetic Stimulation for stress urinary incontinence improves regeneration of skeletal muscle, increases nerve ingrowth and acetylcholine receptor clustering. In: 2012 Annual Meeting of the European Association of Urology.
4. **Meline N L Stölting**; Lukas J Hefermehl; Mathias Tremp, Ryan Khanna, Fahd Azzabi, Tullio Sulser; Daniel Eberli. (2010) *Impact of patient age or gender on bioengineering of functional muscle tissue using Muscle Precursor Cells.* In: 2010 Annual Meeting of the American Urological Association Education and Research Inc.
5. **Meline N L Stölting**; Stefanie Kramer; Simon Ametamey; Stefano Ferrari; Attila Becskei; Tullio Sulser; Daniel Eberli. *Muscle Precursor Cells are safe for the treatment of urinary incontinence after surgery for prostate cancer*
6. **Meline N L Stölting**; Lukas J Hefermehl; Mathias Tremp, Ryan Khanna, Fahd Azzabi, Tullio Sulser; Daniel Eberli. (2010) *Impact of patient age or gender on bioengineering of functional muscle tissue using Muscle Precursor Cells.* In: 2010 Annual Meeting of the American Urological Association Education and Research Inc.
7. Mathias Tremp, MD, **Meline Stölting**, MD, Lukas Hefermehl, MD, Tullio Sulser, MD Daniel Eberli, MD, PhD. (2010) *Contractile function of engineered muscle tissue for the treatment of stress urinary incontinence depends on harvest location.* In: 2010 Annual Meeting of the American Urological Association Education and Research Inc.
8. Lukas J. Hefermehl; **Meline N L Stölting**; Fahd Azzabi.; Tullio Sulser; Daniel Eberli. (2009) *Optimization of human skeletal muscle precursor cell culture and myofibre formation for sphincter reconstruction.* In: 65th Annual Congress of the Swiss Association of Urology.
9. **Meline N L Stölting**; Tullio Sulser; Daniel Eberli. (2009) *Interaction between Adult Muscle Precursor Cells and preexisting Urological Cancer.* In: Brupbacher Symposium 2009 – Targets for Cancer Prevention and Therapy.2009.

10. **Meline N B Lucena.** *Urgências Cirúrgicas em Serviço de Pronto Atendimento (SPA-Alvorada). Descentralização Funcional em uma Unidade Secundária do SUS na Amazônia - Estágio*. 2005. In XLIII Congresso Brasileiro de Educação Médica (XLIII COBEM)
11. **Meline N B Lucena.** *Avaliação da População de Terra Santa – PA quanto à hipertensão Arterial e seus fatores de risco*. 2005. In XLIII Congresso Brasileiro de Educação Médica (XLIII COBEM)
12. **Meline N B Lucena B.** *Perfil Coproparasitológico de crianças ribeirinhas do Lago do Piraruacá, Terra Santa -PA*. 2005. In XLIII Congresso Brasileiro de Educação Médica (XLIII COBEM)
13. **Meline N B Lucena.** *Participação de Acadêmicos de Medicina no Projeto Pé de Pincha (Manejo Sustentável de Quelônios): Relato de Experiência*. 2005. In XLIII Congresso Brasileiro de Educação Médica (XLIII COBEM)
14. **Meline N B Lucena.** *Liga Universitária de Cardiologia e Cirurgia Cardiovascular: Um complemento às atividades Curriculares d Curso Médico*. 2005. In XLIII Congresso Brasileiro de Educação Médica.
15. **Meline N B Lucena**; A. M. L. Oliveira; L. M. C. Nogueira; E. F. Souza; D. B. Ferreira. *Avaliação feita pelos acadêmicos sobre a Infra-estrutura e a logísitica do Curso de Medicina da UFAM – A realidade que o provão não viu.* 2004. In XLI Congresso Brasileiro de Educação Médica (XLI COBEM)
16. M. V. Maia; **Meline N B Lucena**; L. M. C. Nogueira; R. B. Tavares; D. B. Ferreira. *Avaliação dos professores da Medicina da Universidade Federal do Amazonas pelos Estudantes da Graduação – uma análise crítica ao conceito obtido pelo provão*. 2003. In XLI Congresso Brasileiro de Educação Médica.
17. Dirceu B. Ferreira; **Meline N B Lucena**; Lílian M. P. Sakamoto; Lisiane M. C. Nogueira; Edrei F. Souza. *Auto-avaliação Institucional – Modelo Alternativo de Avaliação – Proposta do PET-Medicina da Universidade Federal do Amazonas*. 2003. In XLI Congresso Brasileiro de Educação Médica
18. R. B. Tavares; **Meline N B Lucena**; Lívia Gurgel; Jorge Guerra; D. B. Ferreira. *Estágio – Relato de experiências adquiridas por petianos no setor de Leishmaniose da Fundação de Medicina Tropical do Amazonas – FMT/AM*. 2003. In XLI Congresso Brasileiro de Educação Médica (XLI COBEM)
19. **Meline N B Lucena**; Christopher B. Braun; José A. Alves-Gomes. *Filogeografia de Hypopygus Lepturus (Rhamphichthoidea: Gymnotiformes): testando a hipótese do Rio Negro como barreira de dispersão*. 2003. Anais do PIBIC 2003
20. **Meline N B Lucena**; Christopher B. Braun; José A. Alves-Gomes. *Preliminary mitochondrial DNA diversity analyses of Hypopygus lepturus (Gymnotiformes, Rhamphichthyidae) from the middle Rio Negro.* 2003. Congress of the American Society of Ichthyology and Herpetology, Manaus, Amazonas, Brazil.

Printed: 23.11.2012